DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR CEFIXIME, CEFPODOXIME, CEFEPIME IN BULK AND PHARMACEUTICAL DOSAGE FORM AND ANALYTICAL AND BIOANALYTICAL METHOD FOR BRIVUDINE

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BRANCH–V- PHARMACEUTICAL ANALYSIS

Submitted by

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OCTOBER 2017

Certificate

This is to certify that the dissertation entitled **DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR CEFIXIME, CEFPODOXIME, CEFEPIME IN BULK AND PHARMACEUTICAL DOSAGE FORM AND ANALYTICAL AND BIOANALYTICAL METHOD FOR BRIVUDINE** being submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai was carried out by **C.NAVEEN** in the **Department of Pharmaceutical Analysis,** College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under my direct supervision and guidance to my fullest satisfaction.

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CONTENTS

LIST OF ABBREVIATIONS

HPTLC	High Performance Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
ICH	International Conference on Harmonization
IS	Internal standard
gm	Gram
M	Molar
mM	Millimolar
λmax	Wavelength of maximum absorbance
mg	Milligram
µL	Microliter
µg	Microgram
ng	Nanogram
min	Minute
rpm	Rotation per minute
R_t	Retention time
T_f	Tailing factor
R_s	Resolution
R_f	Retention factor
LOD	Limit of detection
LOQ	Limit of quantification
LLOQ	Lowest limit of quantitation
MLOQ	Middle limit of quantitation
HLOQ	High limit of quantitation
RSD	Relative standard deviation
UV	Ultra violet

INTRODUCTION [1-15]

Pharmaceutical analysis includes both quantitative and qualitative analysis. Qualitative analysis reveals the chemical identification of the species in the sample or yields information about the identification of atomic or molecular species or function groups in the sample. Quantitative analysis establishes the relative amount of one or more of these species, or analytes, in numerical terms.

Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. The official test methods that results from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products.

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods for the quantitation of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Reasons for developing newer analytical methods for drugs:

- Marketed drug may not be official in pharmacopoeias.
- Literature search may not contain complete analytical procedure for such drugs.
- No proper method may be available for drug in the form of formulation due to interference caused by pharmaceutical excipients.
- Procedures for the estimation of drug in biological fluids may not be available.
- Analytical techniques may not be available for the drugs in combination with other drugs.
- Expenses may be more for reagents and solvents used in existing procedures.

CHROMATOGRAPHY

Chromatography was invented and named by the Russian botanist **Mikhail Tsweet** at the beginning of the twentieth century. He employed the techniques to separate various plant pigments such as chlorophylls and xanthophyll by passing solution of these compounds through a glass column packed with finely divided calcium carbonate.

Chromatography is unique in the history of analytical methodology and is probably the most powerful and versatile technique available to modern analyst. In a single procedure it can separate a mixture into its individual components and simultaneously determine quantitatively the amount of each component present.

The samples may be gaseous, liquid or solid in nature and may range in complexity from a single substance to a multicomponent mixture containing widely differing chemical species. Furthermore, the analysis can be carried out, one extreme, on a very costly and complex experiment, and at other, on a simple, inexpensive thin layer plate.

In all chromatography separations the samples is transported in a mobile phase, which may be gas, a liquid, or a supercritical fluid. This mobile phase is then forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface. The two phase are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degrees. Those components that are strongly retained by the stationary phase move only slowly with the flow of mobile phase. In contrast, components that are weakly held by the stationary phase travel rapidly. As a consequences of these differences in mobility, sample components separate into discrete bands, or zones, that can be be analysed qualitatively and or quantitatively.

The chromatographic method of separation, in general, involves the following steps:

- Adsorption
- Separation of the adsorbed substances by the mobile phase.
- Recovery of the separated substances by a continuous flow of the mobile phase; the method being called elution.
- Qualitative and quantitative analysis of the eluted substances

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

HPTLC is a well-known and versatile separation method which shows a lot of advantages in comparison to other separation techniques GC and HPLC as it requires personnel with minimum technical training and not very sophisticated laboratory facilities.

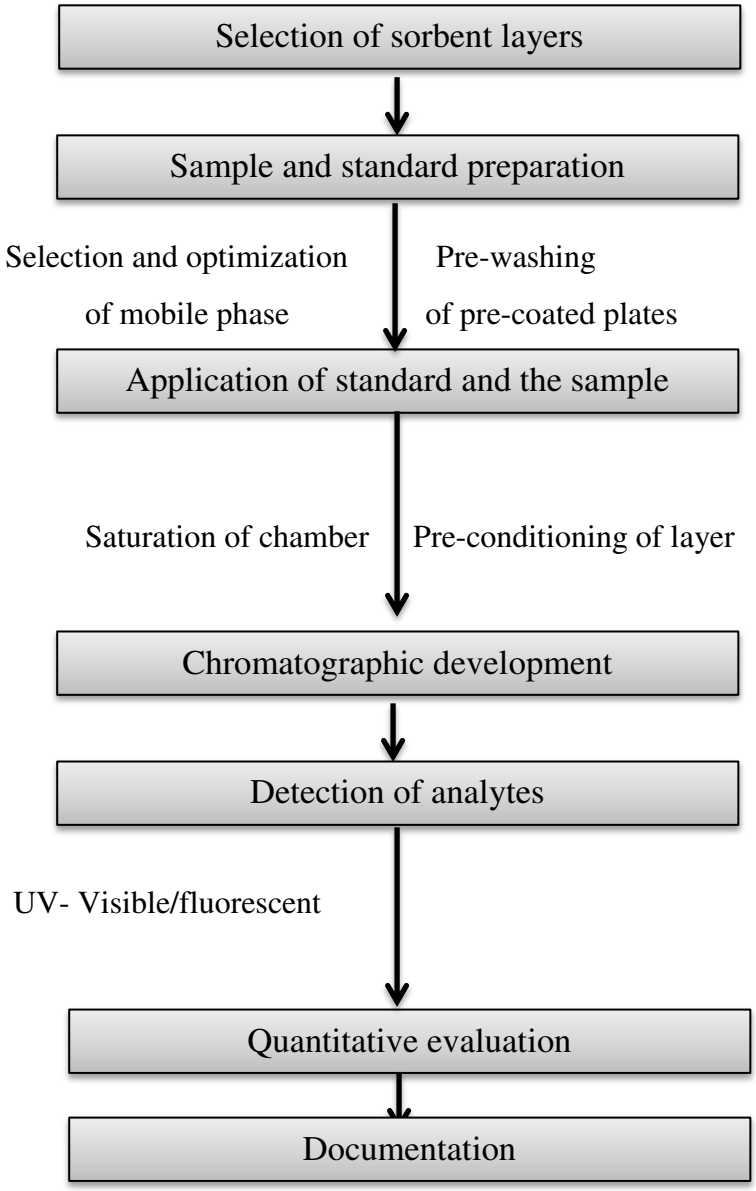
Availability of disposable TLC/HPTLC plates, the problem caused by samples with high matrix contents, which block the HPLC columns and likely to give ghost peaks, is eliminated. HPTLC system is automated and controlled throughout by software and is fully reproducible and standardised cGMP-compliant technique.

Field of application virtually covers all classes of substances such as drugs, pesticides, glycosides, fatty acids, carbohydrates, aflatoxins, metals, foodstuff, environments and clinical samples.

Advantage:

- No cumbersome sample preparation needed as pre-coated plates are disposable.
- Total sample rather than just portion of the sample that elutes from the column (HPLC/GC) occupies the chromatogram(sample integrity).
- Sample components are stored on the plate, allowing repeated analysis.
- Multiple samples can be run simultaneously under identical conditions.
- Similarities and differences are immediately apparent as both sample and standard are applied on the same plate and chromatographic conditions leading to better analytical precision and accuracy.

Various steps involved in HPTLC chromatography



Pre-coated plates

Pre coated plates with different support materials are glass, aluminum, plastic and with different sorbent layers are available in different formats and thickness. Plates with sorbent thickness of 100-250µm are used for qualitative and quantitative analysis.

Pre coated plate contain 1-2% organic polymeric binder. These binders provide smooth abrasive resistant surface and effectively ensure binding of even smallest articles of silica gel to different back materials.

Plate pre-washing:

The TLC plate is fully over run by methanol and dry.

Activation of pre-coated plates

- Freshly open box of plates do not require activation.
- Done by placing in an oven at 110-120ºc for prior to spotting. Aluminum sheets should be kept in between two glass plates and placing in oven at 110-120ºc for 15 minutes.

Application of sample

Usual TLC syringe volume is 100µL. The Linomat V automatic applicator - nitrogen gas sprays. Band wise application-better separation–high response to densitometer.

Selection of mobile phase

Normal phase

- The Stationary phase used is polar and mobile phase is non polar.
- Non polar compounds eluted first because of lower affinity with stationary phase.
- Polar compounds retained because of higher affinity with the stationary phase.

Reversed phase

Stationary phase is non polar, Mobile phase is polar, Polar compounds eluted first because of lower affinity with stationary phase, Non-Polar compounds retained because of higher affinity with the stationary phase.

Pre-conditioning:

a. Chamber saturation has pronounced influence on the separation profile. When the plate is introduced into an unsaturated chamber, the solvent evaporates from the plant mainly at the solvent front.

b. By lining with filter paper prior to development, solvent vapours soon get uniformly distributed throughout the chamber.

c. Twin Trough glass chamber used for plate development.

Detection

- Camag TLC scanner 3
- Detection under UV light is first choice
- Spots of fluorescent compound can be seen at 254nm(short wavelength) or 366nm(long wavelength)
- Densitometric measurement of other planar objective equipped with different lamps (D2 & W).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Liquid chromatography (LC) is the generic name used to describe any chromatographic procedure in which the mobile phase is liquid.

In the early years Liquid chromatography was a slow separation technique performed in vertical columns by gravity flow. Later developments have greatly improved its speed and versatility. The increase in speed has been achieved by pumping the solution through the column at inlet pressure up to and sometime excessing 1000psi. The gains in versatility have come about through the use of smaller diameter, high surface area particles and other unique developments in packing structures and surface. This led to an increase in resolution and introduced the concept of HPLC.

PRINCIPLE

High Performance Liquid chromatography is separation technique where solutes migrate through a column containing a micro-particulate stationary phase at rates dependant on their distribution ratios.

These are functions of the relative affinities of the solute for the mobile and stationary phases, the elution order depending on the chemical nature of the solutes and the overall polarity of the two phases. When a mixture of components dissolved in mobile phases is introduced into the column, the compound with dissolved in mobile phase is introduced into the column, the compound with lesser affinity towards the stationary phase moves faster and hence eluted out of the column first. The one with greater affinity towards the stationary phase moves slower down the column and hence eluted later. Thus the components are separated.

HPLC as compared with the classical LC technique is characterised by

- High resolution. Small diameter (4.6 mm), stainless steel, glass or titanium columns.
- Column packing with very small (3, 5 and 10μm) particles.
- Relatively high inlet pressures and controlled flow of the mobile phase.
- Continuous flow detectors capable of handling small flow rates and detecting very small amounts.
- Rapid analysis.

ISOCRATIC	**GRADIENT**
1. Isocratic, constant eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte-eluent and analyte-stationary-phase interactions are also constant throughout the whole run.	**1**. Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width).
2. This makes isocratic separations more predictable, although the separation power (the number of compounds which could be resolved) is not very high.	**2**. The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width.
3. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak.	**3**. Peak width varies depending on the rate of the eluent composition variation (gradient slope).

BUFFER SELECTION

Choice of buffer is typically governed by the desired pH. The typical pH range for reversed phase on silica-based packing is pH 2 to 8. It is important that the buffer has a pka close to the desired pH since buffer controls pH best at their pka.

General considerations during buffer selection:

- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and possibly selectivity differences).
- Ammonium salts are generally more soluble in organic/water mobile phases·
- TFA can degrade with time, is volatile, absorbs at low UV wavelengths.
- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumulate on column inlets and can damage chromatographic performance.
- At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.

BUFFER CONCENTRATION:

Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed phase HPLC. Phosphate buffers can be replaced with sulfonate buffers when analyzing organophosphate compounds.

Types

Normal phase chromatography

Retention by interaction of the polar surface of stationary phase with polar parts of the sample molecules.

Reverse phase chromatography

Retention by interaction of the nonpolar hydrocarbon chain of stationary phase with nonpolar parts of the sample molecules.

Ion exchange chromatography

Differing affinities of ions in solution for oppositely charged ionic grouping located on the packing. While the nature of the functional group providing the sites for exchange, quaternary ammonium for anions, and sulphonic acid for cations, a variety of substrate ranging from cross linked polystyrene, cross linked polydextrans, cellulose and silica have been utilised.

Ion pair partition

Which contains a counter ion with the opposite charge to that of the ion to be analysed, and which will form an ion pair with the ionic sample components. The counter ion should contain bulky organic substituents so that the ion pair subsequently formed will be hydrophobic in character.

DETECTION:

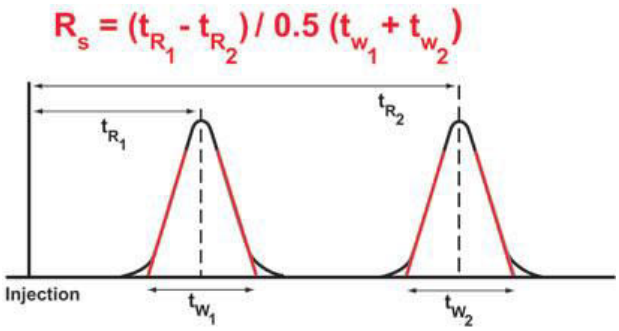
The detection of the separated components in the elute from the column is based upon the bulk property of the elute (eg. Its refractive index) or the solute property of the individual components (eg. Their ultra violet absorption, fluorescence or electrochemical activity). Generally, a detector is selected that will respond to a particular property of the substances being separated, and ideally it should be sensitive to at least 10^{-8} g ml^{-1} and give a linear response over a wide concentration range.

SYSTEM SUITABILITY PARAMETERS:

- **Resolution**
- **Retention time**
- **Tailing factor**
- **Theoretical plates**

Resolution:

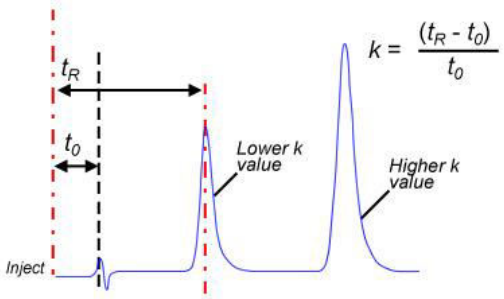
Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular components of the mixture.



The resolution Rs of two neighbouring peaks is defined as the ratio of the distance between two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of Rs is 1.5. it is calculated by using the formula.

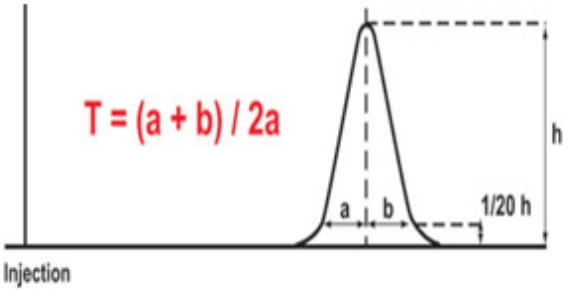
Retention time:

The amount of time between the injection of a sample and its elution from the column is known as the retention time; it is given the symbol $t_{R.}$



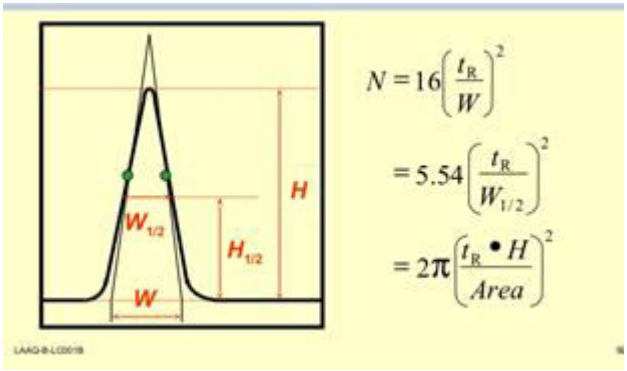
Tailing factor (peak asymmetry factor):

The peak half width, b of a peak at 10% of the peak height, divided by the corresponding front half width, a gives the asymmetry factor.



Theoretical plates (column efficiency):

Efficiency N, of acolumn is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates, including good column and system performance. Column with N ranging from 5000 to 10000 plates/meter are ideal for a good system.



Types of Extraction method

- Liquid -liquid extraction
- Solid phase extraction
- Protein precipitation method

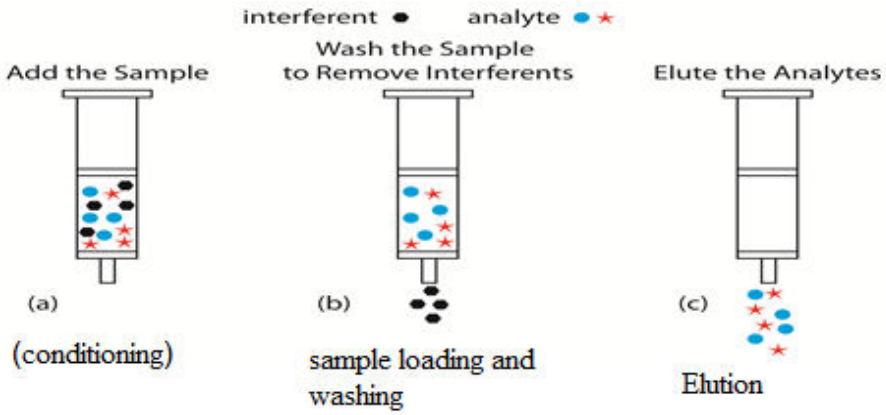
Liquid -liquid extraction

Liquid–liquid extraction (LLE), also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubility's in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid into another liquid phase. It consists of transferring one (or more) solute(s) contained in a feed solution to another immiscible liquid (solvent).

Solid phase extraction:

Solid phase extraction (SPE) is a technique designed for rapid, selective sample preparation and purification prior to chromatographic analysis. Using liquid chromatography principles to control selectivity, SPE provides the sample cleanup, recovery and concentration necessary for accurate quantitative analysis.

Solid-phase extraction (SPE) is a sample preparation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Analytical laboratories use solid phase extraction to concentrate and purify samples for analysis. Solid phase extraction can be used to isolate analytes of interest from a wide variety of matrices, including urine, blood, water, beverages, soil, and animal tissue.



Solid phase extraction cartridges and disks are available with a variety of stationary phases, each of which can separate analytes according to different chemical properties. Most stationary phases are based on silica that has been bonded to a specific functional group. Some of these functional groups include hydrocarbon chains of variable length (for reversed phase SPE), quaternary ammonium or amino groups (for anion exchange), and sulfonic acid or carboxyl groups (for cation exchange).

Protein precipitation method:

This method is least one in bioanalytical. This is a very simple technique for extraction of the analyte from the matrix. If protein binding is suspected, then protein precipitation prior to sample extraction may be considered.

The main requirement for this technique is that the analyte should be freely soluble into reconstituting solvent. Preparation of sample through protein precipitation achieves separation by conversion of soluble proteins to an insoluble state by salting out or by addition of water miscible precipitation solvent or organic solvents such as acetone, ethanol, acetonitrile or methanol.

Commonly the sample is centrifuged at high speed for sufficient time, all the precipitated components of plasma will be settled at the bottom and clear supernatant liquid will be separated out. The obtained supernatant liquid can be injected directly into the HPLC or it can be evaporated and reconstituted with the mobile phase and further cleanup of the sample can be carried out by using micro centrifuge at very high speed.

QUANTITATIVE ANALYSIS

Normalisation:

The Area% calculation procedure reports the area of each peak in the chromatogram as a percentage of the total area of all peaks. Area% does not require prior calibration and does not depend upon the amount of sample injected within the limits of the detector. No response factors are used.

If all components respond equally in the detector and are eluted, then Area% provides a suitable approximation of the relative amounts of components.

External Standard Method:

External standard method involves the use of a single standard or up to three solutions. The peak area or the height of the sample and the standard use are compared directly. One can also use the slope of the calibration curve based on standard that contain known concentration of the compound of interest.

Internal standard Method:

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. A known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatogram to compensate for the losses of the compounds of interest during sample pretreatment steps. The accuracy of this approach obviously dependence on the structural equivalence of the compound of interest and the internal standard.

$$\mathrm{RF} = \frac{\text{standard area or peak height}}{standard\ concentration}$$

STANDARD ADDITION METHOD:

A known amount of the standard compound is added to the ample solution to be estimated. This method is suitable if sufficient amount of the sample is available and is more realize in the sense that it allow calibration in the presence of excipients or other components.

An important aspect of the method of standard addition is that the response prior to spiking additional analyte, it should provide a reasonable S/N ratio(> 10), otherwise the result will have poor precision.

APPLICATIONS OF HPLC

The HPLC has several applications in the fields of pharmacy, forensic, environment and clinical. It also helps in the separation and purification of compound.

Pharmaceutical Applications: The pharmaceutical applications include controlling of drug stability, dissolution studies and quality control.

Environmental Applications: Monitoring of pollutants and detecting components of drinking water.

Forensic Applications: Analysis of textile dyes, quantification of drugs and steroids in biological samples.

Food and Flavour Applications: Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives.

Clinical Applications: Detecting endogeneous neuropeptides, analysis of biological samples like blood and urine.

ANALYTICAL METHOD DEVELOPMENT

Analytical methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long-term stability studies. Methods may also support safety and characterization studies or evaluations of drug performance. According to the International Conference on Harmonization (ICH), the most common types of analytic procedures are:

a. identification tests,
b. quantitative tests of the active moiety in samples of API or drug product or other selected component(s) in the drug product,
c. quantitative tests for impurities' content,
d. Limits tests for the control of impurities.

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The notion of phase-appropriate method development is a critical one if time, cost, and efficiency are concerns. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behavior. They should be suitable to support pre-clinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines.

BIOANALYTICAL METHOD

A bio-analytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound. The most widely accepted guideline for method validation is the ICH guideline Q2 (R1), which is used both in pharmaceutical and medical science.

Bio-analytical method validation (BMV) is the process used to establish that a quantitative analytical method is suitable for biomedical applications. Characterization of the stability of analytes in biological samples collected during clinical studies together with that of critical assay reagents, including analyte stock solutions, is recognized as an important component of bio-analytical assay validation.

Bio-analytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use.

The quality of these studies is directly related to the quality of the underlying Bio-analytical data. It is therefore important that guiding principles for the validation of these analytical methods be established and disseminated to the pharmaceutical community.

Bio-analytical method validation is vital not only in terms of regulatory submission but also for ensuring generation of high quality data during drug discovery and development. BMV assures that the quantification of analyte in biological fluids is reproducible, reliable and suitable for the application.

ANALYTICAL VALIDATION	BIOANALYTICAL VALIDATION
Specificity /selectivity: • Identification test • Determination of impurity • assay	**Selectivity:** • Analyses of blank samples of the appropriate biological matrix should be obtained from at least six sources • Ensured at LLOQ
Accuracy: • Assay - drug substance and drug product • Impurities – samples spiked with known amounts of impurities • Recommended data 1. minimum of 3 concentration levels covering the specified range 2. reported as % recovery	**Accuracy:** • replicate analysis of samples • measured using a minimum of 5 determinations per concentration • mean value within 15% • it should not deviate more than 20%
Precision: • repeatability • intermediate • reproducibility expressed as * standard deviation * relative standard deviation	Precision: • 5 determinations per concentrations • It should not exceed 20% of CV. Division: • Within run precision • Between run precision

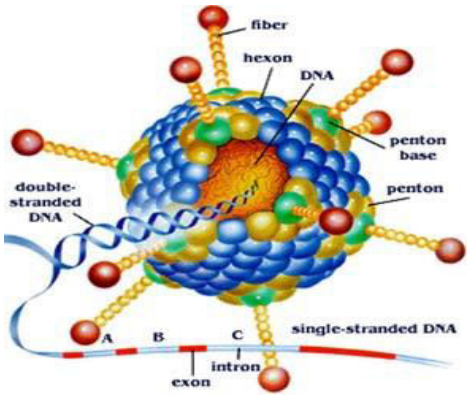
Limit of detection: ➢ Based on visual evaluation ➢ Based on signal to noise ➢ Based on SD of response and slope DL = 3.3 σ/ S σ = standard deviation of response S = slope of calibration curve **Limit of quantitation:** ➢ Based on visual evaluation ➢ Based on signal to noise ➢ Based on SD ○ QL = 10 σ/ S	**Limit of detection:** ➢ The lowest amount of analyte can be detected but not quantified. ➢ The lowest amount of a reference solution that can be detected and others can be detected in biological sample. **Limit of quantitation:** LLOQ: ➢ At least 5 times the response compared to blank ➢ Identifiable, discrete, reproducible, back calculated concentration. ULOQ: ➢ Reproducible and back calculated concentration ➢ Not exceed 15% CV ➢ Accuracy within 15% of nominal concentration.

Linearity: Evaluated by ❖ Visual inspection ❖ Statistical method *minimum 5 concentration I recommended.	Linearity: ❖ Directly proportional to the concentration of the analyte in the sample. ❖ 5 concentrations levels should bracket the upper and lower concentration levels evaluated during study.
Robustness: ▪ It should show the reliability of an analysis with respect to deliberate variations in method parameters. ▪ pH ▪ m/p ratio ▪ temperature, ▪ flow rate	**Robustness:** ▪ To measure for the susceptibility of a method to small chances that might during routine analysis. **Stability:** ▪ Long term stability ▪ Freeze/ thaw stability ▪ In process stability ▪ Processed sample stability.

HERPES ZOSTER

Herpes zoster is a skin rash that occurs due to varicella zoster virus (the same virus that causes chicken pox). A person acquires chicken pox usually during childhood, which is a self-limiting disease most of the time. This virus then remains dormant in nerve cells of the body for months to years and may again reactivate during adulthood to cause herpes zoster also known as **shingles.**

Herpes zoster occurs in patients who have a decrease in body's resistance power. With decreasing immunity, the virus reactivates, begins to multiply and then moves along the nerve fibres towards the skin. It is commonly seen in elderly people as their immune system weakens. Other patients at risk are cancer patients on chemotherapy or radiotherapy, patients with organ transplant and HIV infected patients.



2-3 days prior to the skin rash, patients may have burning pain or tingling in one area of the skin, which is usually on one side of body. This is followed by appearance of blisters on a red base again in same area of skin. These blisters last for two to three weeks, during which pus may appear, then they crust and finally begin to disappear leaving behind a depigmented area on the skin. The pain may last longer and sometimes pain is severe enough to require painkillers.

Brivudine is an analogue of the nucleoside thymidine. The active compound is brivudine 5'-triphosphate, which is formed in subsequent phosphorylations by viral (but not human) thymidine kinase and presumably by nucleoside-diphosphate kinase. Brivudine 5'-triphosphate works because it is incorporated into the viral DNA, but then blocks the action of DNA polymerases, thus inhibiting viral replication.

Brivudine is used for the treatment of herpes zoster in adult patients. It is taken orally once daily, in contrast to aciclovir, valaciclovir and other antivirals. A study has found that it is more effective than aciclovir.

REVIEW OF LITERATURE

The need of survey of literature is to see what has and has not been investigated and identify data sources that other researchers have used. To learn how others have defined and measured key concepts and develop alternative research projects. Review of literature helps to demonstrate understanding and ability to critically evaluate research in the field and to provide evidence that may be used to support one's own findings.

Pritam Jain *et al.,* (2012) reported stability indicating HPTLC method for determination of cefpodoxime proxetil in bulk and tablet dosage form. The chromatographic conditions used for separation was silica gel 60 RP-18 f_{254} and mobile comprised of toluene: methanol: chloroform (4: 2: 4 v/v). The R_f value was found to be 0.55. The linearity was found to be 100-600ng/band for cefpodoxime with correlation coefficient of 0.998. The method was successfully applied to pharmaceutical formulation.

Laxman Prajapati *et al.,* (2016) have developed a new simple, precision, sensitive and validated HPTLC method for simultaneous estimation of cefepime hydrochloride and sulbactum sodium.the chromatographic conditions are Silica Gel $G_{60}F_{254}$ HPTLC Plates using the mobile phase of chloroform: ethyl alcohol: Diethyl amine: water (12:7:1:0.4V/V). The Retention Factor ($R_{f)}$ was found to be 0.17 for cefepime hydrochloride and 0.76 for sulbactam sodium respectively. Cefepime hydrochloride and sulbactam sodium showed linear response at concentration range 4-20 µg/band and 2-10 µg/band. The Correlation co-efficient (r2) for cefepime hydrochloride and sulbactam sodium was found to be 0.9997 and 0.9997 respectively.

AIM AND OBJECTIVE

Cefixime, Cefpodoxime, Cefepime are antibiotic drugs under the class of cephalosporin antibiotics. These drugs are used to treat respiratory tract infections, skin infections and urinary tract infections.

Brivudine is an antiviral drug (herpes zoster virus). The FDA approved brivudine June 29, 2011 to treat shingles ((herpes zoster).

Literature reveals very few analytical techniques for the estimation of cefpodoxime and cefepime from tablets. Also there is no HPTLC analytical method reported for the cefixime.

Literatures have proven that there are no analytical method reported for the brivudine in formulation as well as biofluids like plasma.

Hence the major objective of the present research work is,

- Development and validation of HPTLC method for determination of cefixime, cefpodoxime, and cefepime in bulk and pharmaceutical dosage form.
- Development and validation of HPTLC and HPLC method for estimation of brivudine in bulk and pharmaceutical dosage form.
- Development and validation of bioanalytical method for the estimation of brivudine in human plasma.

DRUG PROFILE[18-21]

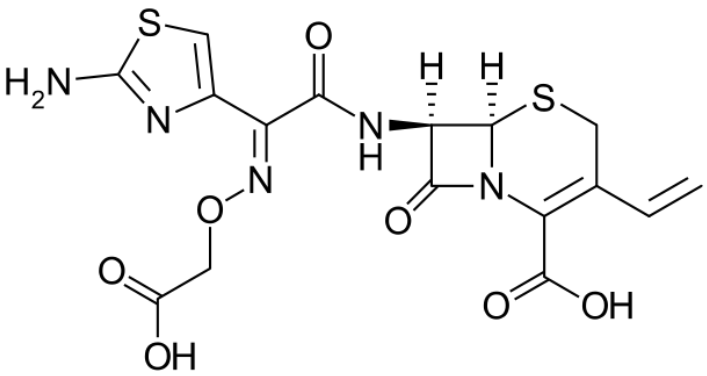
CEFIXIME

Name : Cefixime

IUPAC Name : (6R,7R)-7-[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-[(carboxymethoxy)imino]acetamido]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

Empirical Formula : $C_{16}H_{15}N_5O_7S_2$

Structure Formula :



Molecular Weight : 453.452 g/mol

Description : white powder

Solubility : methanol, water

Melting point : 218-225 °C

Bioavailability : 40% - 50%

Protein Binding : 60%

Storage : Refrigerator 25°C

Category : Antibiotic

Action and use **:** The bactericidal action of Cefixime is due to the inhibition of cell wall synthesis. It binds to one of the penicillin binding proteins (PBPs) which inhibits the final transpeptidation step of the peptidoglycan synthesis in the bacterial cell wall, thus inhibiting biosynthesis and arresting cell wall assembly resulting in bacterial cell death.

Available dosage forms : Tablet, syrup, suspension, powder, Capsule

Brands : AIM-200, cefi D S, cemax, Penixime, suprax

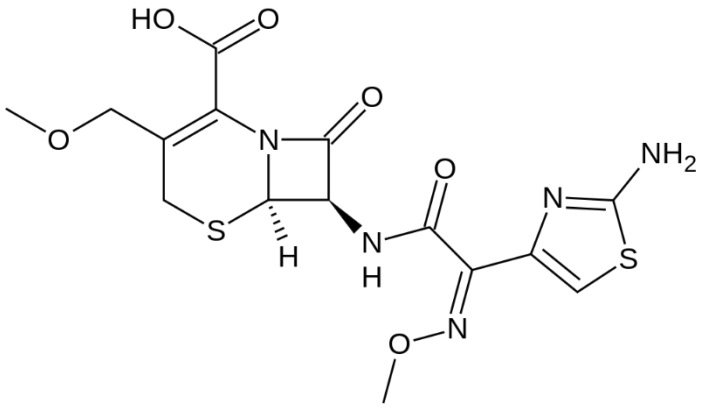
CEFPODOXIME PROXETIL

Name : Cefpodoxime proxetil

IUPAC Name : (6R,7R)-7-[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-(methoxyimino)acetamido]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid

.**Empirical Formula** : $C_{15}H_{17}N_5O_6S_2$

Structure Formula :



Molecular Weight : 427.58g/mol

Description : white powder

Solubility : methanol, DMSO

Melting point : 111-113^{o}C

Bioavailability : 50 %

Protein Binding : 21-29%

Storage : 25^{o}C

Category : Antibiotic Agent

Action and use **:** Cefpodoxime is a bactericidal agent that acts by inhibition of bacterial cell wall synthesis. Cefpodoxime has activity in the presence of some beta-lactamases, both penicillinases and cephalosporinases, of Gram-negative and Gram-positive bacteria.

Available dosage forms : Tablet , suspension

Brands : ALTIPOD 200, BALPOD

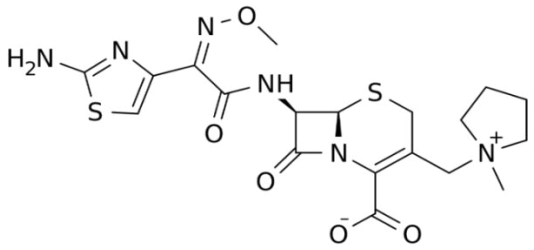
CEFEPIME

Name : cefepime

IUPAC Name : 1-{[(6R,7R)-7-[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-(methoxyimino)acetamido]-2-carboxylato-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl}-1-methylpyrrolidin-1-ium

Empirical Formula : $C_{19}H_{24}N_6O_5S_2$

Structure Formula :



Molecular Weight : 450.56 g/mol

Description : white powder

Solubility : methanol, DMSO

Melting point : 111-113^{o}C

Bioavailability : 100 %

Protein Binding : 15%

Storage : 25^{o}C

Category : Antibiotic Agent

Action and use : Cephalosporins exert bactericidal activity by interfering with bacterial cell wall synthesis and inhibiting cross-linking of the peptidoglycan. The cephalosporins are also thought to play a role in the activation of bacterical cell autolysins which may contribute to bacterial cell lysis.

Available dosage forms : Powder for injection

Brands : cefudix, cefwin, Biopime, C-pime

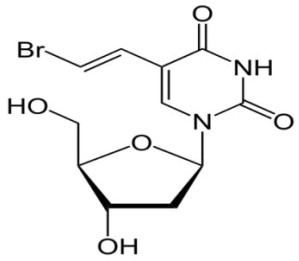
BRIVUDINE

Name : Brivudine

IUPAC Name : 5-[(E)-2-bromoethenyl]-1-[(2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]pyrimidine-2,4-dione

.Empirical Formula : $C_{11}H_{13}BrN_2O_5$

Structure Formula :



Molecular Weight : 333.135 g/mol

Description : White powder

Solubility : Soluble in methanol, DMSO

Melting point : 165-166^{o}C

Bioavailability : 30 %

Protein Binding : >95%

Storage : 25^{o}C

Category : Antiviral Agent

Action and use Brivudine is an analogue of the nucleoside thymidine. The active compound is brivudine 5'-triphosphate, which is formed in subsequent phosphorylations by viral (but not human) thymidine kinase and presumably by nucleoside-diphosphate kinase.

Brivudine 5'-triphosphate works because it is incorporated into the viral DNA, but then blocks the action of DNA polymerases, thus inhibiting viral replication.

Available dosage forms : Tablet

Brands : Zostex

MATERIALS AND INSTRUMENTS

CHEMICALS AND SOLVENTS USED

⊗ Water for HPLC

⊗ Methanol HPLC grade, AR grade

⊗ Ortho phosphoric acid AR grade

⊗ Triethyl amine AR grade

⊗ Distilled water

All the above chemicals and solvents were supplied by S.D. Fine Chemicals Ltd., India, Qualigens Fine Chemicals Ltd., Mumbai, India, Sigma-aldrich Chemicals Pvt. Ltd., Maharashtra, India.

MATERIALS USED

- Pre-coated silica gel $60F_{254}$ on aluminium sheets were procured from Merck, Germany.
- Phenomenex luna, C_{18} column (250mm×4.0mm, 5µm).

INSTRUMENTS USED

- Shimadzu digital electronics balance
- pH meter Elico Pvt. Limited, India.
- Camag HPTLC system (with TLC Scanner-3, Win CATS software and Linomat 5 as application device)
- Shimadzu HPLC system with SPD-M10 A VP system PDA with 20µl fixed volume manual injector and LC-MS solution software.
- Jasco V-630 spectrophotometer with a pair of 1 cm quartz Cuvette.

EQUIPMENTS USED

- Sonicator (Leelasonic ultrasonic sonicator)
- Centrifuge (Eppendorf Mini Spin)
- Borosil micro pipet (AUTOCLAVABLE)

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR DETERMINATION OF CEFIXIME IN BULK AND PHARMACEUTICAL DOSAGE FORM

Selection of plate:

Pre-coated silica gel $G_{60}F_{254}$ on aluminium sheet was selected for the study.

Selection of solvent:

Methanol was selected as a solvent because cefixime is soluble in methanol.

Selection of wavelength:

The sensitivity of HPTLC method depends upon the proper selection of wavelength for UV detection. An ideal wavelength is the one that give maximum absorbance and good response for the drug to be detected at the lower concentration.

Selection of optimum mobile phase

The selection of the mobile phase is perhaps the most important parameter to achieve efficient thin layer chromatography separation. A solvent system that would give dense compact spot and good separation from solvent front and application position was to be selected. Initially different solvent systems were tried and observations were as given below:

Solvent system	Observation
Toluene: ethyl acetate: methanol (4.5: 4.5: 1 % v/v/v)	**Spot moving but not clear spot**
Toluene: ethyl acetate: methanol (0.5: 1.5: 8 % v/v/v)	**Drug moving along with solvent front**
Ethyl acetate: Methanol: water (4:6:1 % v/v/v)	**Higher R_f value**
Ethyl acetate: Methanol: water (4.5: 5: 0.5 %v/v)	**Compact spot**

Among these systems, **Ethyl acetate: Methanol: water** was selected because this system gave good symmetric peak.

Optimization of chamber saturation time

The above fixed mobile phase was added to one side of a twin trough chamber and different saturation times from 10 to 20 minutes were tried. It was found that a saturation time of more than 15 minutes caused fluctuations in the R_f value with edge effect. Hence a saturation time of 15 minutes was fixed for further studies.

Fixed experimental conditions

Stationary phase : Pre-coated silica gel $G_{60}F_{254}$aluminium sheets
Mobile phase : Ethyl acetate: Methanol: water (4.5: 5: 0.5 %v/v)
Chamber saturation : 15 minutes
Migration distance : 80 mm
Band width : 6 mm
Slit dimension : 5 × 0.45 mm
Source of radiation : Deuterium lamp
Scanning wave length : 292 nm
R_f value of cefixime : 0.58 ± 0.02

VALIDATION OF THE METHOD

The validation of the developed method was carried out in terms of linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter and intra-day precision and stability studies as per ICH guidelines.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by applying decreeasing amount of the drug in triplicate on the plate. The lower concentration at which the peak is detected is called limit of detection. The lowest concentration at which the peak is qunantified is called limit of quantification.

Linearity and range

A 100µg/ml solution of cefixime was prepared in methanol. Aliquots of 2, 2.5, 3, 3.5, 4, and 4.5µl of cefixime were applied on the plate. The plate was developed, scanned and peak areas were noted. Linear regression data showed a good correlation coefficient over a concentration range of 200-450 ng/band.

Accuracy

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. It was done by mixing known quantities of the standard drug with the analysed sample formulation and the contents were reanalysed by proposed method. Recovery studies were carried out at 80% and 120% levels.

Precision

Precision of method was demonstrated by:

i. Intra-day precision
ii. Inter-day precision
iii. Repeatability

a) Repeatability of sample application
b) Repeatability of sample measurement

Intra-day precision

Intraday precision was studied by different concentration in linearity range of the drug for several times on the same day and by calculating % RSD.

Intra-day precision:

Intraday precision was studied by standard drug at two concentrations in the linearity range of the drug for three days over a period of one week and % RSD was calculated.

Repeatability:

Repeatability of sample application was carried by spotting 3.5µl of drug solution is six times on pre coated TLC plate followed by development of plate and % RSD was calculated.

Repeatability of measurement was determined by spotting 3.5µl of drug solution on a pre-coated TLC plate and developed the plate and scanned six times and % RSD was calculated.

Robustness:

The robustness of the method is its ability to remain unaffected by small change in practical conditions. Here the effect of change in condition such as ratio of mobile phase (±0.5%) and saturation time (±5min) were studied to prove robustness.

Stability of chromatographic plate:

When the developed chromatographic plate is exposed to atmosphere the analyte are likely to decompose. Hence it is necessary to study the stability of drug on plate. It was studied by scanning the plate at different time intervals and peak areas were compared with the peak area of freshly scanned plate. The developed plate was found to be stable for about 8 hours for cefixime.

ANALYSIS OF FORMULATION

Twenty tablets each containing 200mg of cefixime were taken and the average weight was calculated. They were finely pulverized and the quantity of homogenised powder equivalent to 10mg of cefixime was transferred to a 100ml volumetric flask and up to volume with methanol. Then the solution was filtered using whattmann filter paper followed by application of the solution on pre coated TLC plate. After development the plate was scanned at 292nm and the peak area were noted. The amount of cefixime present in each tablet was calculated and tabulated.

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR DETERMINATION OF CEPODOXIME PROXETIL IN BULK AND PHARMACEUTICAL DOSAGE FORM

Selection of plate:

Pre-coated silica gel $G_{60}F_{254}$ on aluminium sheet was selected for the study.

Selection of solvent:

Methanol was selected as a solvent because cefpodoxime is soluble in methanol.

Selection of wavelength:

The sensitivity of HPTLC method depends upon the proper selection of wavelength for UV detection. An ideal wavelength is the one that give maximum absorbance and good response for the drug to be detected at the lower concentration.

Selection of optimum mobile phase

The selection of the mobile phase is perhaps the most important parameter to achieve efficient thin layer chromatography separation. A solvent system that would give dense compact spot and good separation from solvent front and application position was to be selected. Initially different solvent systems were tried and observations were as given below:

Solvent system	Observation
Toluene: ethyl acetate : methanol (4.5: 4.5 : 1 % v/v/v)	**Drug moved but not clear spot**
Toluene: ethyl acetate : methanol (4:4:2 % v/v/v)	**Drug moved along with solvent front**
Methanol: Ethyl acetate: toluene (1.5:3:5.5 %v/v)	**Compact spot**

Among these systems, **Ethyl acetate: Methanol: toluene** was selected because this system gave good symmetric peak.

Optimization of chamber saturation time

The above fixed mobile phase was added to one side of a twin trough chamber and different saturation times from 10 to 20 minutes were tried. It was found that a saturation time of more than 15 minutes caused fluctuations in the R_f value with edge effect. Hence a saturation time of 15 minutes was fixed for further studies.

Fixed experimental conditions

Stationary phase	:	Pre-coated silica gel $G_{60}F_{254}$ aluminium sheets
Mobile phase	:	Methanol: Ethyl acetate: toluene (1.5:3:5.5 %v/v)
Chamber saturation	:	15 minutes
Migration distance	:	80 mm
Band width	:	6 mm
Slit dimension	:	5 × 0.45 mm
Source of radiation	:	Deuterium lamp
Scanning wave length	:	280 nm
R_f value of cefpodoxime	:	0.53 ± 0.02

VALIDATION OF THE METHOD

The validation of the developed method was carried out in terms of linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter and intra-day precision and stability studies as per ICH guidelines.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by applying decreeasing amount of the drug in triplicate on the plate. The lower concentration at which the peak is detected is called limit of detection. The lowest concentration at which the peak is qunantified is called limit of quantification.

Linearity and range

A 100µg/ml solution of cefpodoxime was prepared in methanol. Aliquots of 1, 1.5, 2, 2.5and 3µl of cefpodoxime were applied on the plate. The plate was developed, scanned and peak areas were noted. Linear regression data showed a good correlation coefficient over a concentration range of 100-300 ng/band.

Accuracy

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. It was done by mixing known quantities of the standard drug with the analysed sample formulation and the contents were reanalysed by proposed method. Recovery studies were carried out at 80% and 120% levels.

Precision

Precision of method was demonstrated by:

- Intra-day precision
- Inter-day precision

Repeatability

- Repeatability of sample application
- Repeatability of sample measurement

Intra-day precision

Intraday precision was studied by different concentration in linearity range of the drug for several times on the same day and by calculating % RSD.

Intra-day precision:

Intraday precision was studied by standard drug at two concentrations in the linearity range of the drug for three days over a period of one week and % RSD was calculated.

Repeatability:

Repeatability of sample application was carried by spotting 3.5µl of drug solution is six times on pre coated TLC plate followed by development of plate and % RSD was calculated.

Repeatability of measurement was determined by spotting 3.5µl of drug solution on a pre-coated TLC plate and developed the plate and scanned six times and % RSD was calculated.

Stability of chromatographic plate:

When the developed chromatographic plate is exposed to atmosphere the analyte are likely to decompose. Hence it is necessary to study the stability of drug on plate. It was studied by scanning the plate at different time intervals and peak areas were compared with the peak area of freshly scanned plate. The developed plate was found to be stable for about 8 hours for cefpodoxime.

ANALYSIS OF FORMULATION

Twenty tablets each containing 200mg of cefpodoxime were taken and the average weight was calculated. They were finely pulverized and the quantity of homogenised powder equivalent to 10mg of cefpodoxime was transferred to a 100ml volumetric flask and up to volume with methanol. Then the solution was filtered using whattmann filter paper followed by application of the solution on pre coated TLC plate. After development the plate was scanned at 292nm and the peak area were noted. The amount of cefpodoxime present in each tablet was calculated and tabulated.

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR DETERMINATION OF CEFEPIME IN BULK AND PHARMACEUTICAL DOSAGE FORM

Selection and pre washing of plate

A pre coated silica gel $G_{60}F_{254}$ on aluminium sheet was selected for the study. Pre washing o the plate was done with methanol and then it was activated by keeping in an oven at 115^{o}C for 10 mins.

Selection of wavelength

The sensitivity of HPTLC method depends upon the proper selection of wavelength of UV detection. An ideal wavelength is that the one that give maximum absorbance and good response for the drug to be detected at lower concentration. The spectrum of cefepime was shown λ_{max} at 285nm. Hence it was selected as the detection wavelength.

Development of optimum mobile phase

A solvent system that would give dense compact spots and good separation from solvent front and application position was to be selected for the study. Initially, different solvents system were tired and observations were as given below in table 1.

Solvent system	Observation
methanol : water: chloroform (8: 1: 1 % v/v/v)	**No movement of spots was observed**
Ethyl acetate: ethanol: methanol (6:2:3 % v/v/v)	**No movement of spots was observed**
Toluene: acetone: methanol (6;3.5;0.5)	**No movement of spots was observed**
Methanol: water: ethanol (4: 1.5: 2 % v/v/v)	**Drug moved along with the solvent front**
methanol : water: chloroform (7: 2: 1 % v/v/v)	**Drug moved but not clear spot**
methanol : water: chloroform (6: 3: 1 % v/v/v)	**Compact spot**

Optimization of chamber saturation

The above fixed mobile phase was added to one side of twin trough chamber and different saturation times from 5 to 20min were tried. It was found that a saturation time of less than 10min caused fluctuation in R_f value and edge effect. Hence a saturation of 15min was fixed.

Fixed experimental conditions

Stationary phase	:	Pre-coated silica gel $G_{60}F_{254}$ aluminium sheets
Mobile phase	:	Methanol: water: chloroform (6: 3: 1 % v/v/v)
Chamber saturation	:	15 minutes
Migration distance	:	80 mm
Band width	:	6 mm
Slit dimension	:	5 × 0.45 mm
Source of radiation	:	Deuterium lamp
Scanning wave length	:	285 nm
R_f value of cefepime	:	0.44

VALIDATION OF THE METHOD

The validation of the developed method was carried out in terms of linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter and intra-day precision and stability studies as per ICH guidelines.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by applying decreeasing amount of the drug in triplicate on the plate. The lower concentration at which the peak is detected is called limit of detection. The lowest concentration at which the peak is qunantified is called limit of quantification.

Linearity and range

A 100µg/ml solution of cefixime was prepared in methanol. Aliquots of 5, 10, 15, 20, and 25µl of cefixime were applied on the plate. The plate was developed, scanned and peak areas were noted. Linear regression data showed a good correlation coefficient over a concentration range of 500-2500ng/band.

Accuracy

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. It was done by mixing known quantities of the standard drug with the analysed sample formulation and the contents were reanalysed by proposed method. Recovery studies were carried out at 80% and 120% levels.

Precision

Precision of method was demonstrated by:

- Intra-day precision
- Inter-day precision

Repeatability

- Repeatability of sample application
- Repeatability of sample measurement

Intra-day precision

Intraday precision was studied by different concentration in linearity range of the drug for several times on the same day and by calculating % RSD.

Intra-day precision:

Intraday precision was studied by standard drug at two concentrations in the linearity range of the drug for three days over a period of one week and % RSD was calculated.

Repeatability:

Repeatability of sample application was carried by spotting 15µl of drug solution is six times on pre coated TLC plate followed by development of plate and % RSD was calculated.

Repeatability of measurement was determined by spotting 15µl of drug solution on a pre-coated TLC plate and developed the plate and scanned six times and % RSD was calculated.

Robustness:

The robustness of the method is its ability to remain unaffected by small change in practical conditions. Here the effect of change in condition such as ratio of mobile phase (±0.5%) and saturation time (±5mins) were studied to prove robustness.

Stability of chromatographic plate:

When the developed chromatographic plate is exposed to atmosphere the analyte are likely to decompose. Hence it is necessary to study the stability of drug on plate. It was studied by scanning the plate at different time intervals and peak areas were compared with the peak area of freshly scanned plate. The developed plate was found to be stable for about 24 hours for cefepime.

ANALYSIS OF FORMULATION

Ten formulation of Powder for injection (1g/10ml) of cefepime was taken and the average weight was calculated. They were finely pulverized and the quantity of homogenised powder equivalent to 10mg of cefepime was transferred to a 100ml volumetric flask and up to volume with methanol. Then the solution was filtered using whattmann filter paper followed by application of the solution on pre coated TLC plate. After development the plate was scanned at 285nm and the peak area were noted. The amount of cefepime present in each tablet was calculated and tabulated.

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF BRIVUDINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

Selection of plate:

Pre-coated silica gel $G_{60}F_{254}$ on aluminium sheet was selected for the study.

Selection of solvent:

Methanol was selected as a solvent because Brivudine is soluble in methanol.

Selection of wavelength:

The sensitivity of HPTLC method depends upon the proper selection of wavelength for UV detection. An ideal wavelength is the one that give maximum absorbance and good response for the drug to be detected at the lower concentration.

Selection of optimum mobile phase

The selection of the mobile phase is perhaps the most important parameter to achieve efficient thin layer chromatography separation. A solvent system that would give dense compact spot and good separation from solvent front and application position was to be selected. Initially different solvent systems were tried and observations were as given below:

Solvent system	Observation
Methanol : Chloroform(3:7 v/v)	**Drug moved along with solvent front**
Methanol : Chloroform(4:6 v/v)	**Drug moved along with solvent front**
Methanol : Chloroform(1:9 v/v)	**Higher Rf value**
Methanol:Chloroform: toluene (2:5:3v/v/v)	**Compact spot**

Among these systems, **Methanol: Chloroform: toluene** was selected because this system gave good symmetric peak.

Optimization of chamber saturation time

The above fixed mobile phase was added to one side of a twin trough chamber and different saturation times from 10 to 20 minutes were tried. It was found that a saturation time of more than 15 minutes caused fluctuations in the R_f value with edge effect. Hence a saturation time of 15 minutes was fixed for further studies.

Fixed experimental conditions

Stationary phase : Pre-coated silica gel 60 F_{254} aluminium sheets
Mobile phase : Methanol: Chloroform: toluene (2:5:3v/v/v)
Chamber saturation : 15 minutes
Migration distance : 80 mm
Band width : 6 mm
Slit dimension : 5 × 0.45 mm
Source of radiation : Deuterium lamp
Scanning wave length : 301 nm
R_f value of brivudine : 0.50 ± 0.02

VALIDATION OF THE METHOD

The validation of the developed method was carried out in terms of linearity, accuracy, limit of detection (LOD), limit of quantification (LOQ), inter and intra-day precision and stability studies as per ICH guidelines.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined by applying decreeasing amount of the drug in triplicate on the plate. The lower concentration at which the peak is detected is called limit of detection. The lowest concentration at which the peak is qunantified is called limit of quantification.

Linearity and range

A 100µg/ml solution of brivudine was prepared in methanol. Aliquots of 0.5, 1, 1.5, 2, 2.5 and 3µl of brivudine were applied on the plate. The plate was developed, scanned and peak areas were noted. Linear regression data showed a good correlation coefficient over a concentration range of 50-300 ng/band.

Accuracy

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. It was done by mixing known quantities of the standard drug with the analysed sample formulation and the contents were reanalysed by proposed method. Recovery studies were carried out at 80% and 120% levels.

Precision

Precision of method was demonstrated by:

- Intra-day precision
- Inter-day precision

Repeatability

- Repeatability of sample application
- Repeatability of sample measurement

Intra-day precision

Intraday precision was studied by different concentration in linearity range of the drug for several times on the same day and by calculating % RSD.

Intra-day precision:

Intraday precision was studied by standard drug at two concentrations in the linearity range of the drug for three days over a period of one week and % RSD was calculated.

Repeatability:

Repeatability of sample application was carried by spotting 2µl of drug solution is six times on pre coated TLC plate followed by development of plate and % RSD was calculated.

Repeatability of measurement was determined by spotting 14µl of drug solution on a pre-coated TLC plate and developed the plate and scanned six times and % RSD was calculated.

Robustness:

The robustness of the method is its ability to remain unaffected by small change in practical conditions. Here the effect of change in condition such as ratio of mobile phase (±1% of methanol) and saturation time (±3min) were studied to prove robustness.

Stability of chromatographic plate:

When the developed chromatographic plate is exposed to atmosphere the analyte are likely to decompose. Hence it is necessary to study the stability of drug on plate. It was studied by scanning the plate at different time intervals and peak areas were compared with the peak area of freshly scanned plate. The developed plate was found to be stable for about 8 hours for brivudine.

ANALYSIS OF FORMULATION

Twenty tablets each containing 125mg of brivudine were taken and the average weight was calculated. They were finely pulverized and the quantity of homogenised powder equivalent to 10mg of brivudine was transferred to a 100ml volumetric flask and up to volume with methanol. Then the solution was filtered using whattmann filter paper followed by application of the solution on pre coated TLC plate. After development the plate was scanned at 301nm and the peak area were noted. The amount of brivudine present in each tablet was calculated and tabulated.

DEVELOPMENT OF VALIDATION RP-HPLC METHOD FOR ESTIMATION OF BRIVUDINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

Selection of stationary phase:

Brivudine is a polar drug, hence in the HPLC method a column C18 was used for the separation and estimation of brivudine from tablet dosage form.

Selection of solvent:

The drug was readily soluble in methanol and showed good stability. So methanol was selected as solvent.

Selection of wavelength:

Good analytical separation can be obtained only by careful selection of wavelength for the detection. This choice requires knowledge of the UV spectrum of the sample component. A UV spectrum of brivudine was recorded in methanol which showed in maximum absorbance at 254nm and it was selected for the study.

OPTIMIZATION OF CHROMATOGRAPHY CONDITIONS:

Optimization of mobile phase

Optimization of mobile phase was carried out in order to obatain ideal peak of brivudine.

The mobile phase system consisting of ortho phosphoric acid and methanol was optimized for their and pH of the buffer.

Effect of ratio:

The effect of ratio was studied by varying strength such as 45:55, 50:50,40:60, 60:40,65:35.From which a ratio of 40:60 was selected for further study.

Effect of pH:

Keeping the ratio of mobile phase constant (40:60 % v/v) the chromatograms were recorded at different pH between 3.4 –7. A good resolution and symmetrical peak was obtained at a pH of 6.5 and selected for further studies.

FIXED CHROMATOGRAPHY CONDITIONS

Chromatographic method	:	RP-HPLC
Stationary phase	:	phenomenox luna, C18 column (250mm*4.0mm,5µ)
Mobile phase	:	1% O-phosphoric acid: methanol
Ratio of mobile phase	:	40: 60
Detection wavelength	:	254nm
Flow rate	:	0.8ml/min
Retention time	:	5.1

Validation of the method

The development HPLC method was validated according to ICH guidelines in term of specificity, linearity, accuracy, precision, robustness and LOD and LOQ.

Specificity:

It was studied by injecting the mobile phase, and found that no additional peaks were appeared and no interference at the retention time of brivudine. The peak purity index was also noted.

Linearity and range:

From the stock solution of brivudine (100mcg/ml), standard solutions were prepared in the concentration range 0.5-3mcg/ml in methanol. These solutions were injected into HPLC system and chromatograms were recorded. The peak areas of these chromatograms were measured at 254nm. A calibration graph was plotted with measured concentration against peak areas. The slope, intercept, and coefficient values were calculated.

Accuracy:

To confirm accuracy of the method a known quantity of standard drug was added with the preanalysed formulation and the contents were reanalyzed by the proposed HPLC method. It was carried out at 80 & 120% level.

Precision:

The precision of the method was determined by performing

- Intra – day precision
- Inter-day precision
- Repeatability

Intraday precision:

Intraday precision was determined by injecting standard solution in between linearity range 1.5mcg/ml & 2mcg/ml were injected three times on the same day and % RSD was calculated.

Interday precision:

Interday precision was determined by injecting standard solution in between linearity range 1.5mcg/ml & 2mcg/ml were injected for three days and % RSD was calculated.

Repeatability:

Repeatability of injection was determined by injecting standard solutions 1mcg/ml for six times, noted peak areas and % RSD was calculated.

Limit of detection and limit of quantitation:

LOD & LOQ were calculated in terms of signal to noise ratio. LOD is the lowest concentration of the analyte that can produce a response detectable above the noise level of the system, typically, three times the noise level.

Robustness:

In order to determine the robustness of the method, slight change were made in the practical condition, like flow rate, organic solvent and pH. The standard brivudine was injected and observed in each condition.

Stability of solution:

The standard solution of brivudine was kept under room temperature. It was injected periodically, stability was studied by looking for any change in retention time, resolution and peak shape, when compared to chromatograms of freshly prepared solutions. The solution was stable for hours under room temperature.

System suitability parameters:

The system suitability parameters like peak area, tailing factor, theoretical plate count, resolution, and retention time were calculated from the standard chromatograms.

ANALYSIS OF FORMULATIONS

Twenty tablets of each tablet containing 125mg of brivudine were taken for the study and average weight was determined. The powder of tablets equivalent to 10mg of brivudine was weighed and transferred to 100ml standard flask and it was dissolved in small portion of methanol, then the volume was made up. it was filtered and further dilutions were made and injected into fixed chromatographic system and the peak area was measured from which the amount present/tablet was calculated.

DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD FOR THE ESTIMATION OF BRIVUDINE IN HUMAN PLASMA

Chromatographic conditions:

The HPLC system for brivudine consist of mobile phase is 1% O-phsophoric acid: Methanol (40:60), stationary phase is C_{18} and detection wavelength at 254nm. The RT of brivudine was observed at 5.1min.

Preparation of standard stock solution:

The stock solution of 100µg/ml brivudine was prepared by dissolving in methanol and stored volumetric flask. Standard working solution of brivudine was prepared containing the concentrations of 0.5mcg/ml-3mcg/ml of brivudine.

OPTIMIZATION OF EXTRACTION PROCEDURE:

Selection of internal standard:

To select internal standard (IS) different drugs (100mcg/ml of each) like lamivudine, acyclovir, ledipasvir, sofosbuvir, diclofenac sodium, aspirin were tired. Sofosbuvir sodium was selected as it gave symmetrical peak and had good resolution from brivudine.

Optimization of extraction solvent and volume:

The protein precipitation technique was preferred to extract brivudine from plasma. Based on solubility of two organic solvents, such as methanol, DMSO were tried to achieve maximum recovery of brivudine from spiked plasma. Also different volumes like 0.1ml, 0.2ml, 0.3ml, 0.5ml were of each organic solvent was tried to optimize procedure.

Fixed extraction procedure:

To a clean and dry centrifuge tube (eppendrof tube), 100 µl of human plasma and 100 µl of aliquots of standard drug solution (0.5mcg/ml-3mcg/ml) were added and vortexed for 2minutes and 100 µl of internal standard was added and vortexed for 3minutes and 1ml of methanol was added to above the solution and vortexed for 5minutes. This solution was centrifuged at 1000 rpm for 15minutes. The clear supernatant solution (20 µl) was filtered through syringe membrane filter and injected in optimized chromatographic condition.

VALIDATION OF THE METHOD

Specificity:

Specificity of the above developed extraction method was demonstrated as follows. The blank plasma samples were extracted using the optimized procedure and chromatogram was recorded. It was observed for any interference at retention time of drug and internal standard peak.

Linearity range:

Linear regression data was studied between concentration and peak area ratio of drug to internal standard over a concentration range of 0.5mcg/ml-3mcg/ml. the standard curve covers the entire range of expected concentration. The slope, intercept, and correlation coefficient values were calculated.

Accuracy:

The accuracy of the method was studied at three concentrations in the range of 0.5mcg/ml-3mcg/ml. It was observed if the mean value is within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy.

Recovery:

An important parameter associate with bioanalytical methods is recovery. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. The extraction efficiency was explained by recovery studies. The standard drug was extracted by the proposed method. The extracts were analyzed with the fixed chromatographic conditions. The peak was compared with that of unextracted standard peak areas. The peak area response obtained was used for determining extraction efficiency. Recovery should be determined by using three concentration levels. The % extraction or % recovery was calculated by using the followed formula,

$$Absolute\ recovery = \frac{response\ of\ analyte\ spiked\ into\ matrix(processed)}{response\ of\ analyte\ of\ pure\ standad\ (unprocessed)} X\ 100$$

Precision:

The precision of the method was carried out at three different concentrations in expected concentration range. The precision determined at each concentration level if it is not exceeding 15% coefficient of variation (CV) except for the LOQ where it should not exceed 20% CV. Precision may be considered at three levels: repeatability, inter-day precision, intra-day precision.

$$\%CV = \frac{standard\ deviation}{mean} \times 100$$

Intra-day precision:

Intra-day precision was studied the standard drug at three different concentrations in the linearity range of drug (high QC, middle QC, and low QC) for five times same day and coefficient of variation was calculated.

Inter-day precision:

Inter-day precision was studied the standard drug at three different concentrations in the linearity range of drug (high QC, middle QC, and low QC) for five times same day and coefficient of variation was calculated.

Lower limit of quantitation:

The lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy.

Middle limit of quantification:

The middle concentration of analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy.

Higher limit of quantitation:

The higher concentration of analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy.

Stability:

The aim of a stability test is to detect any degradation of the analytes of interest during the entire period of sample collection, processing, storing, preparing, and analysis. The condition under which the stability is determined is largely dependent on the nature of the analyte, the biological matrix, and the anticipated time period of storage. Stability should be confirmed for every step of sample preparation and analysis, as well as the conditions used for long-term storage.

Short-term stability

The stability of the analyte in biological matrix at ambient temperature should be evaluated. Three aliquots of low and high concentration should be kept for at least 24 hours and then analysed.

The stability of the drug was studied under the room temperature.

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF CEFIXIME IN BULK AND PHARMACEUTICAL DOSAGE FORM

Among various mobile phase system tried the one contain a mixture of ethyl acetate: methanol: water (4.5: 5: 0.5 % v/v/v) gave compact dense band was chosen for the estimation of cefixime by HPTLC method. The plate used was pre-coated silica gel $G_{60}F_{254}$. The method was for the validated and details are given below.

Linearity and range:

A 100µg/ml of cefixime was prepared in methanol. Aliquots of this solution (2-4.5µl) were applied on the plate. The plate was developed, scanned and peak areas were noted. Linear regression data showed good correlation coefficient over a concentration range of (200-450ng/band). The calibration graph is shown in fig 1. The slope, intercept, and correlation coefficient values were in noted (table 1). The spectra of band obtained was recorded on HPTLC scanner (fig. 2) and the standard densitograms are shown in fig.3 to 8, and calibration data was shown table 2.

Fig 1 Calibration graph of cefixime (200-450ng/band)

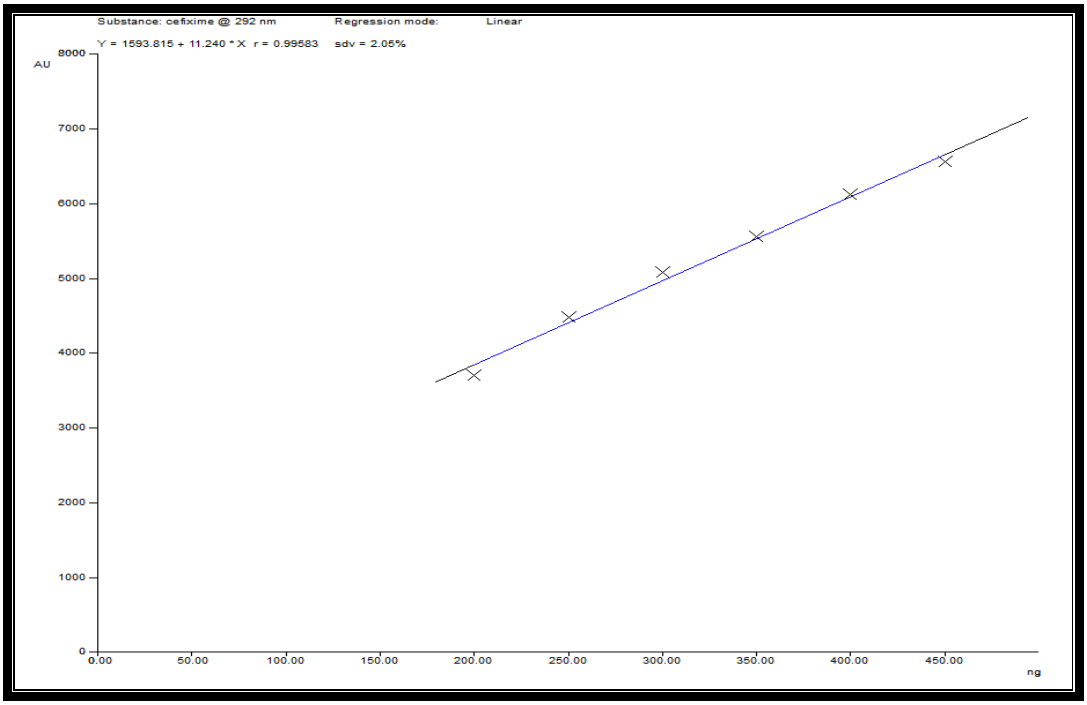


Table 1: Regression data of cefixime

Linear regression	Cefixime
Slope	11.21
Intercept	593.815
Correlation coefficient	0.998

Table 2: calibration data for cefixime

Concentration (ng/band)	Peak area
200	3286.5
250	3704.8
300	4095.9
350	4561.2
400	4900.4
450	5368.1

Fig.2 spectrum of cefixime band

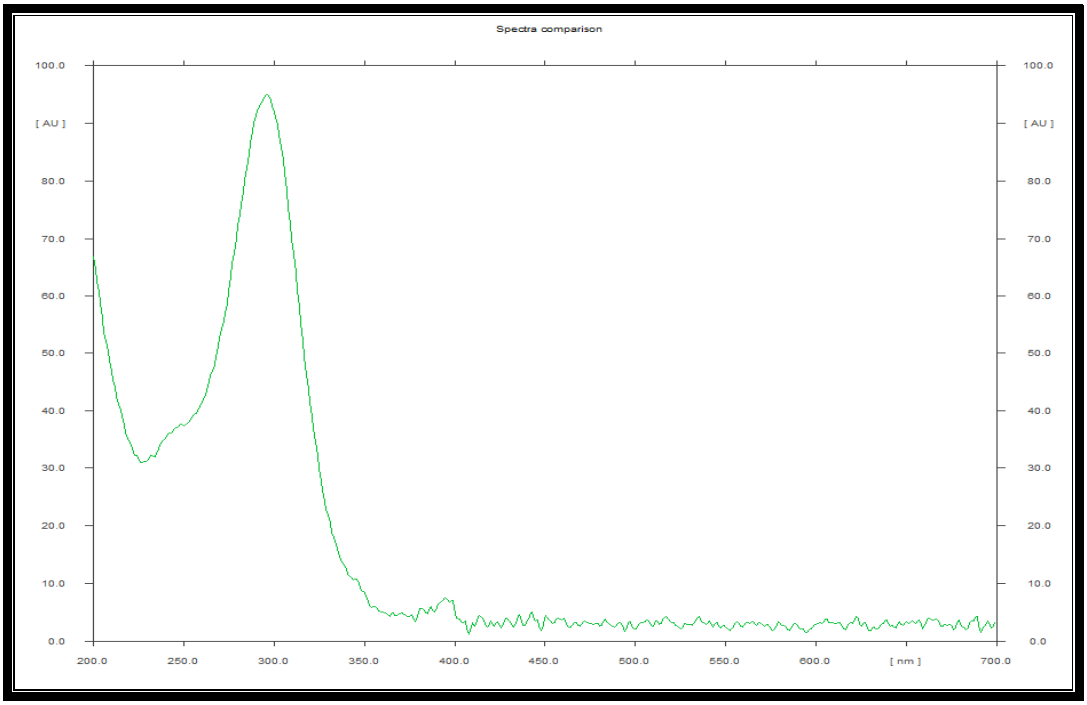


Fig. 3 Densitogram of cefixime 200ng/band

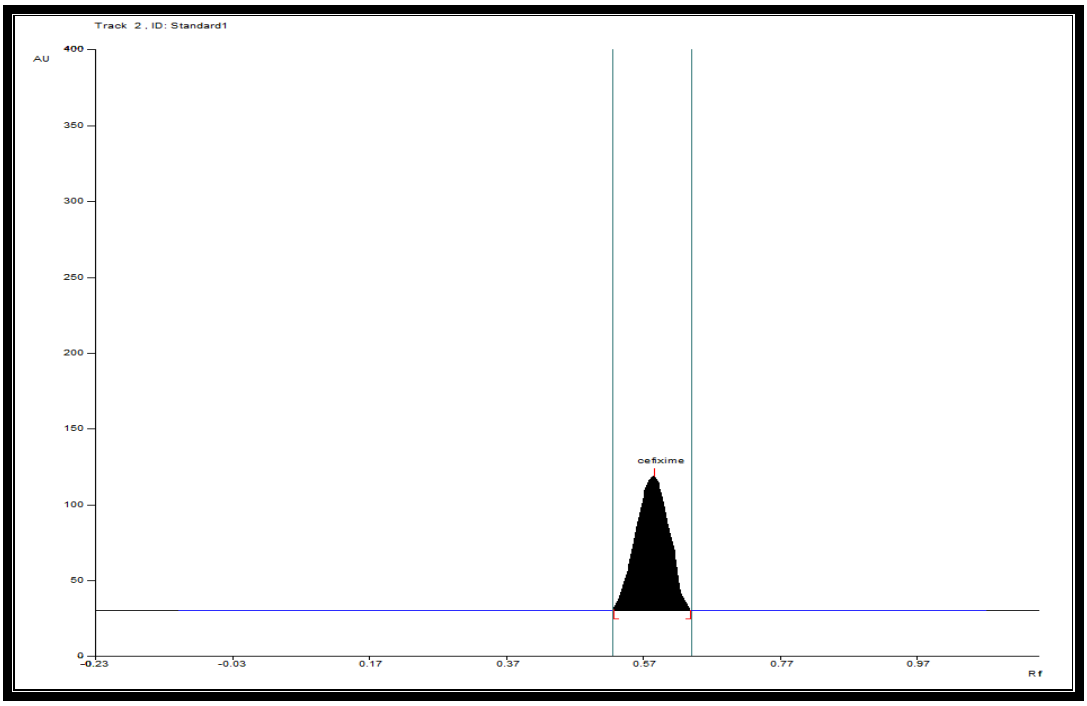


Fig. 4 Densitogram of cefixime 250ng/band

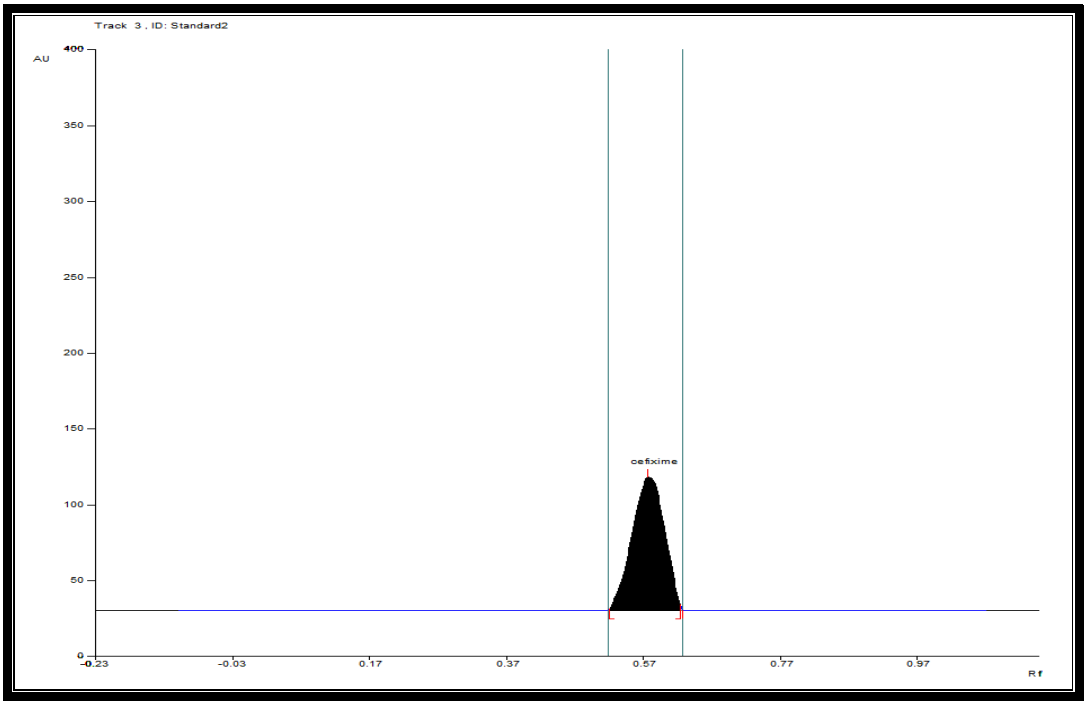


Fig. 5 Densitogram of cefixime 300ng/band

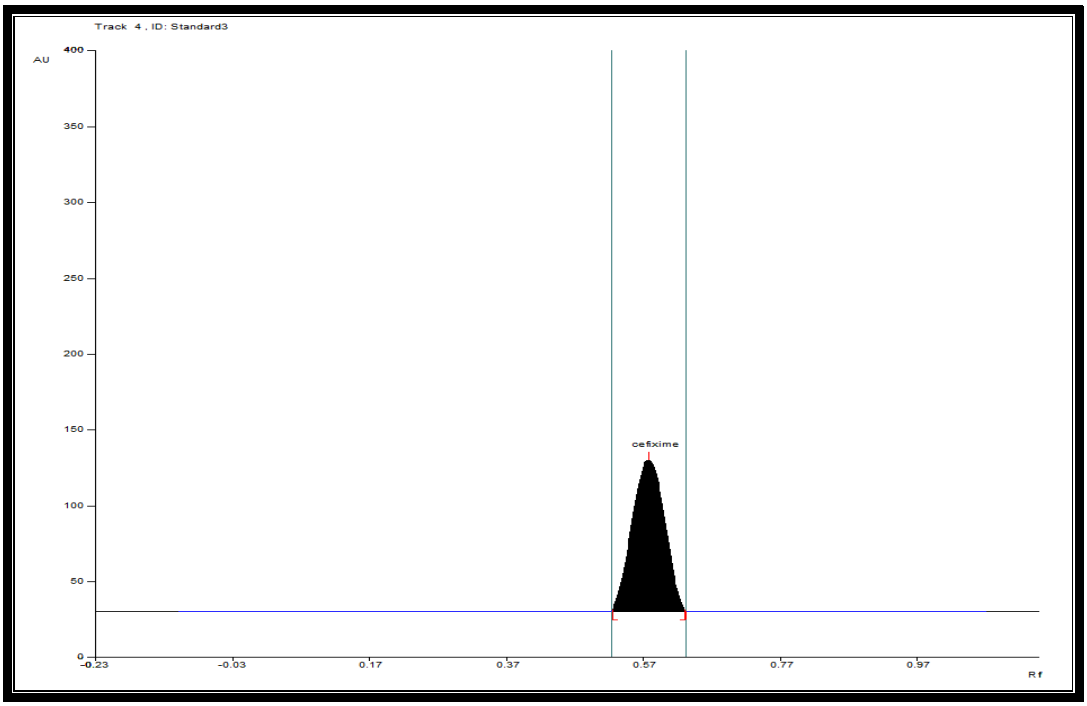


Fig .6 Densitogram of cefixime 350ng/band

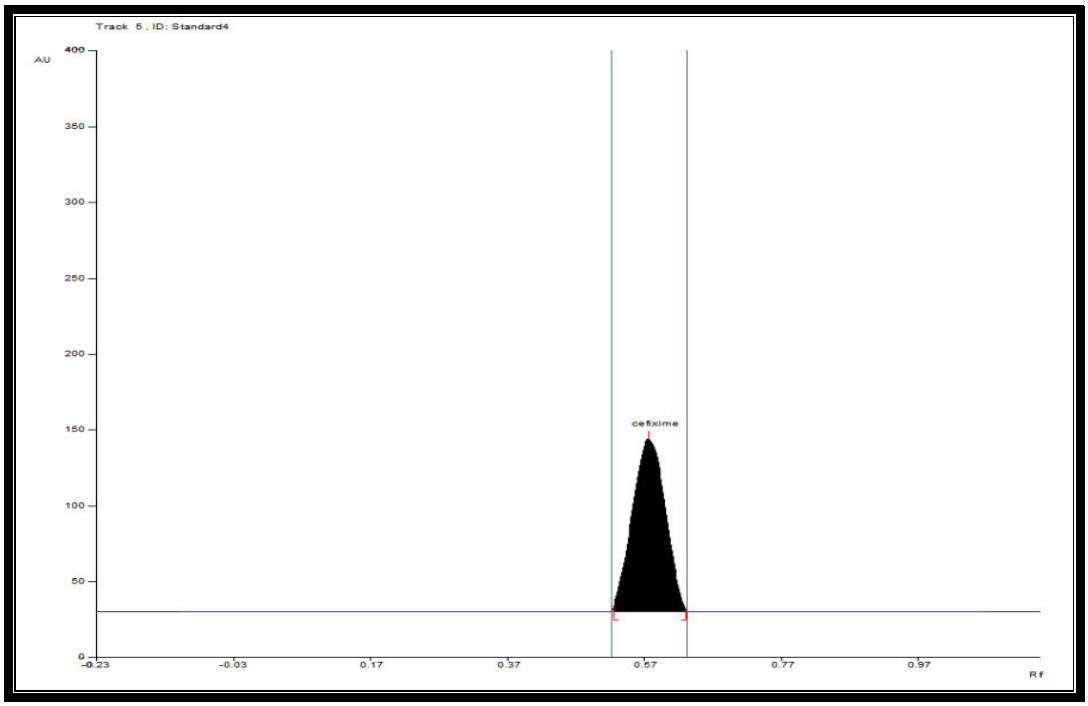


Fig.7 Densitogram of cefixime 400ng/band

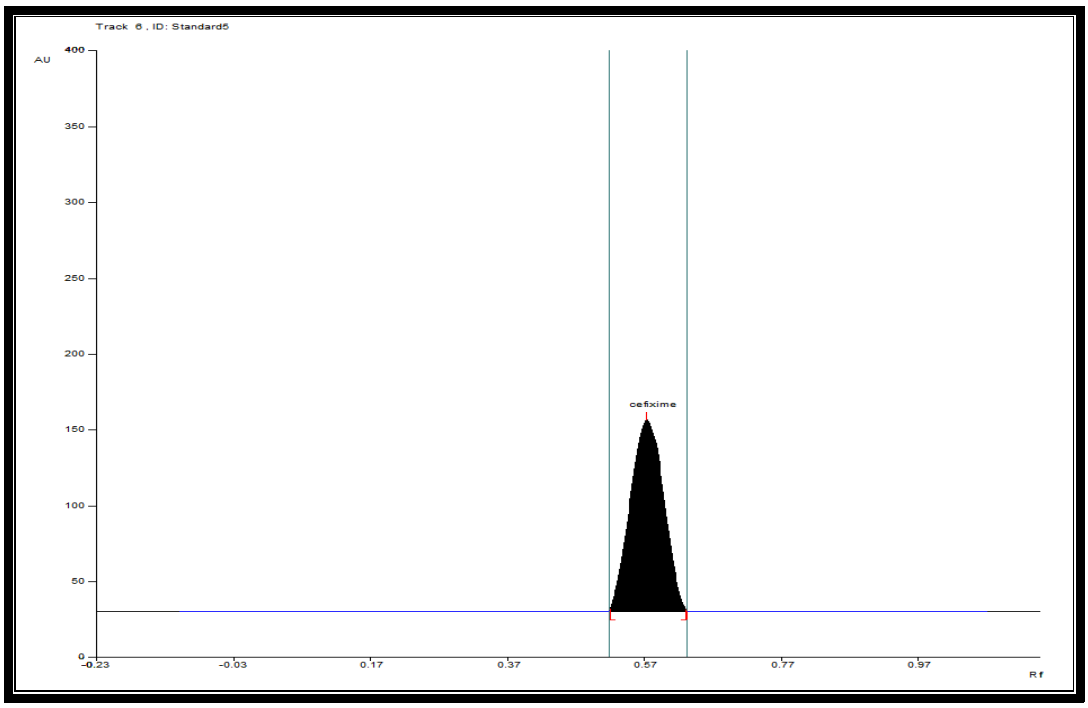
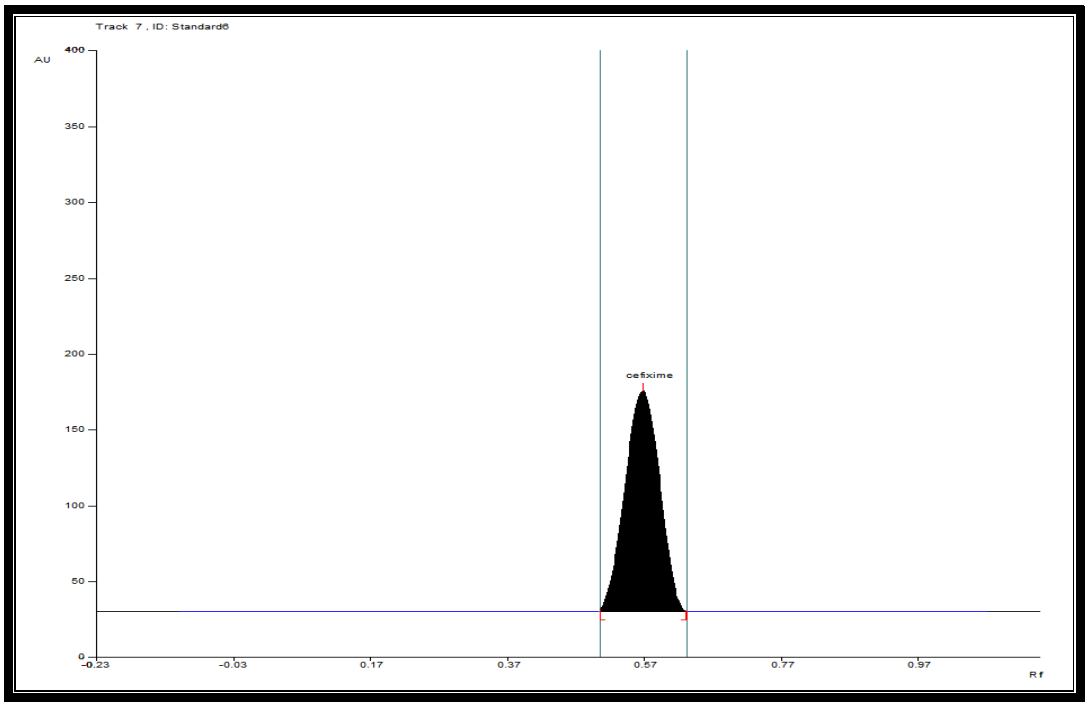


Fig. 8 Densitogram of cefixime 450ng/band



Precision

The method precision was obtained by repeating the determination of standard drug of two selected concentrations (350 & 400ng/band) of cefixime. The % RSD was calculated for inter-day, intra-day and repeatability (repeatability of sample measurement and sample application respectively) are shown in table 3 & 4.

Table 3: Intraday and interday precision

Concentration (ng/band)	Peak area		% RSD	
	Intraday	Interday	Intraday	Interday
350	4466.8	4279.8	0.9	1.9
	4544.2	4328.3		
	4483.6	4431.2		
400	5318.6	5278.9	0.2	1.4
	5341.8	5169.4		
	5316.9	5278.7		

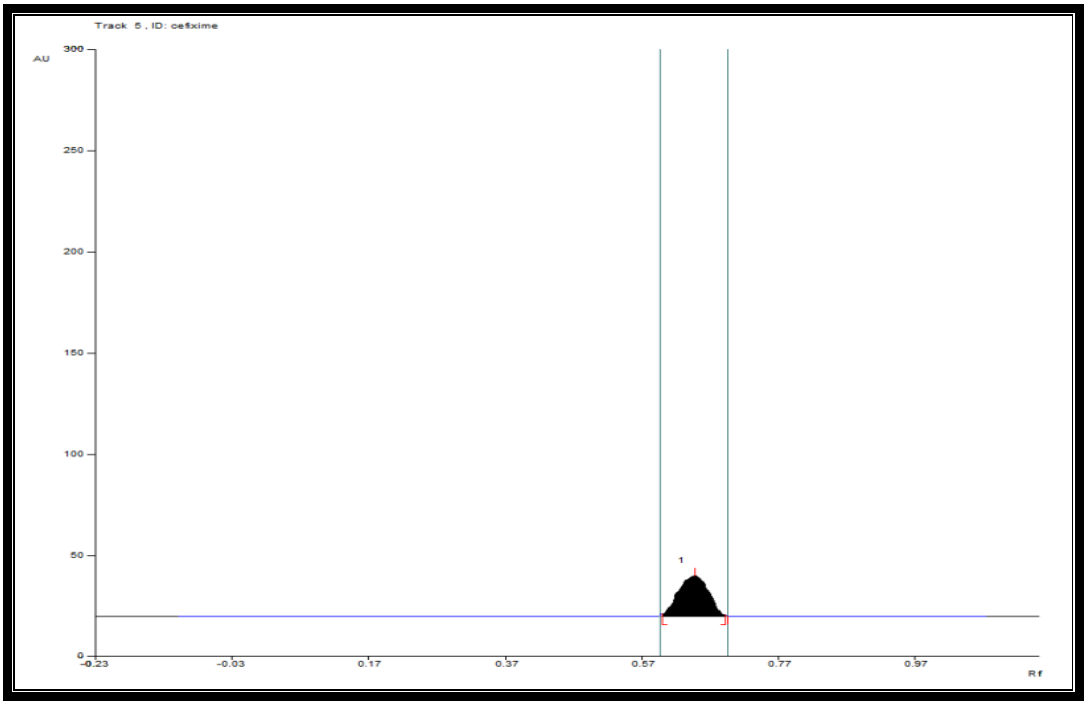
Table 4: Repeatability sample measurement and sample application

Concentration (ng/band)	Peak area		%RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
400	5396.8	5298.6	0.6	0.9
	5378.6	5369.8		
	5310.0	5241.0		
	5318.6	5364.9		
	5341.8	5360.1		
	5316.9	5300.1		

Limit of detection (LOD) and limit of quantitation (LOQ):

The lowest concentration of the analyte detectable was found to be 40ng/band (fig. 9). The lowest concentration of the analyte at which it is quantifiable was found to be 100ng/band.

Fig.9 LOD of cefixime 40ng/band



Robustness

The robustness of the method is its ability to remain unaffected by small changes in the parameter such as ratio of mobile phase and saturation time. The method is said to be robust as minor variation (as shown below) not affected quantification.

Parameters	Observation
Ratio of mobile phase ± 0.5 methanol (ethyl acetate: methanol: water)	Slight changes in R_f, but no difference in peak area
Saturation time(15 ± 2minutes)	Peak symmetry retained.

Stability of chromatographic plate

When the developed method chromatographic plate was exposed to atmosphere, the analytes are likely to decomposed. Hence it was necessary to conduct stability studies of the plate.

Stability of the analyte on the plate was studied at different time intervals and peak areas were compared with peak area of freshly scanned plate. The developed plate was found to be stable for less than 24hrs (table 5).

Table 5: Stability of chromatographic plate

Time (hr)	Concentration(ng/band)	Peak area
0	250 300	3289.7 3820.1
8	250 300	3189.2 3879.3
24	250 300	3096.1 3450.0

ANALYSIS OF FORMULATION

20 tablets, each containing 200mg of cefixime were taken for the study and average weight was determined. Quantity equivalent to10mg of cefixime was weighed and transferred to 100ml volumetric flask added 10ml methanol and sonicated. It was centrifuged for 30min and made up to volume with methanol. It was filtered and used for analysis.

Recording chromatogram

With the fixed chromatographic condition, a suitable volume of sample solutions was applied on the precoated TLC plate. The plate was analyzed and chromatogram was recorded (fig 10). The UV spectrum of cefixime recorded on scanner is shown in fig 11. Peak areas of sample chromatograms were noticed and the amount of drugs was calculated, table 6.

Fig. 10 Densitogram of Cefixime 250ng/band

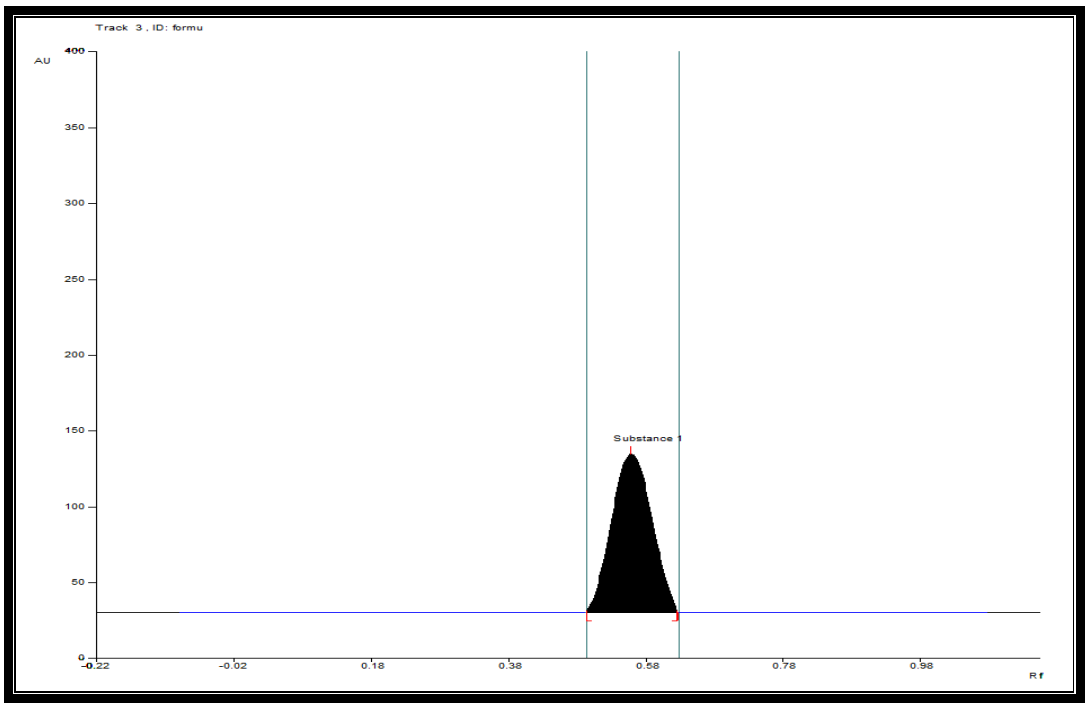


Fig. 11: Spectrum of cefixime band

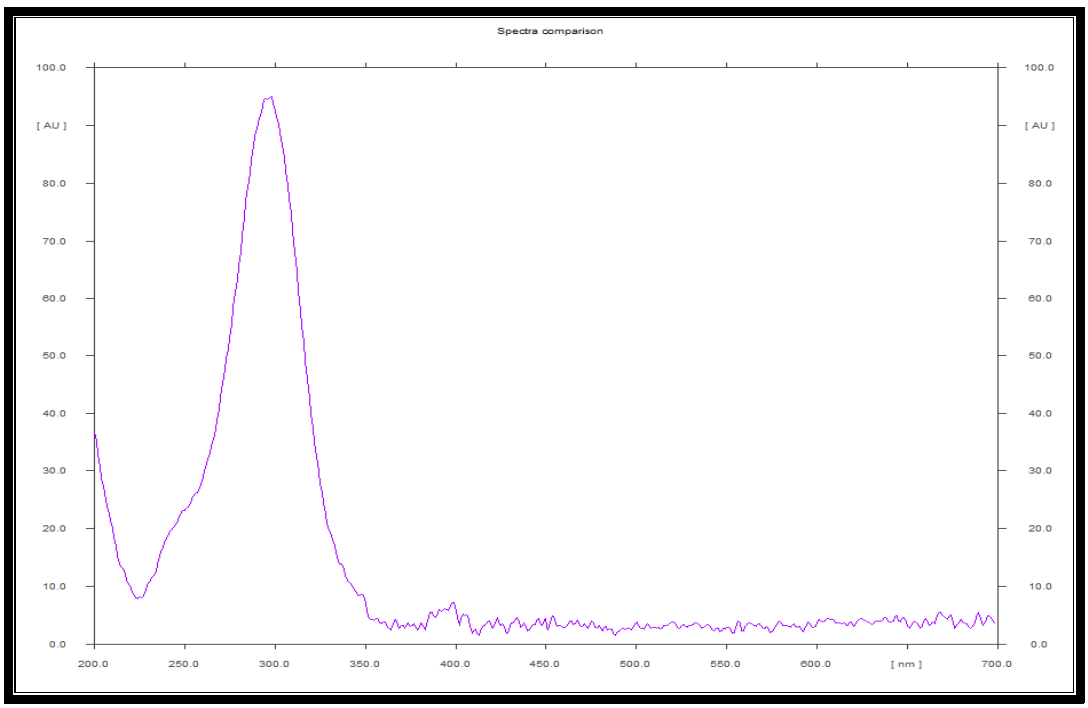


Table 6: Results analysis of formulation

Brand name	Amount of drug/tablet		%label claim	%RSD*
	Labeled (mg/ tablet)	Found (mg/ tablet)		
ALTIPOD 200	200	195.2	97.6%	0.6

***average of six observations**

Recovery:

Recovery studies were carried out at 80% & 120% levels. The percentage recovery and percentage % RSD of the results were calculated and shown in table 7. The value proves the accuracy of the method.

Table 7: Recovery study

Levels	% Recovery	%RSD*
80	99.81%	0.87
120	100.9%	0.78

*Average of six observation

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR DETERMINATION OF CEFPODOXIME IN BULK AND PHARMACEUTICAL DOSAGE

Different mobile phase compositions are tried for development and validation of cefpodoxime by HPTLC method. Among these mobile phase system, methanol: ethyl acetate: toluene (1.5:3:5.5 %v/v) was selected because in this system compact and dense band with good separation were obtained.

Linearity and range:

Linear regression data showed a good correlation coefficient over a concentration range 100-300ng/band. The calibration data are shown in the table 8 and the slope, intercept and correlation coefficient values were found are presented in table 9 respectively. The calibration graph is shown in fig 12. The spectrum recorded on HPTLC scanner (fig 13) and the standard chromatograms obtained at different concentrations of cefpodoxime are shown in fig. 14-18.

Table. 8 calibration data of cefpodoxime

Concentration(ng/band)	Peak area
100	1230.4
150	1538.6
200	1894.2
250	2184.0
300	2551.3

Table 9: Regression data of cefpodoxime

Linear regression	Cefpodoxime
Slope	8.124
Intercept	293.581
Correlation coefficient	0.999

Fig. 12 Calibration graph of cefpodoxime

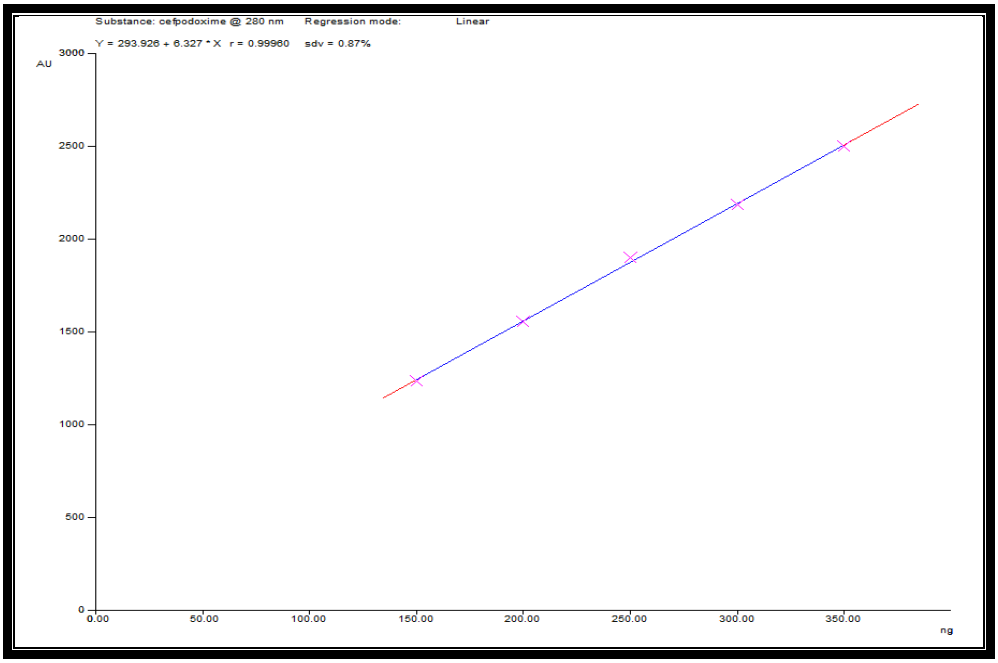


Fig. 13spectrum of cefpodoxime band

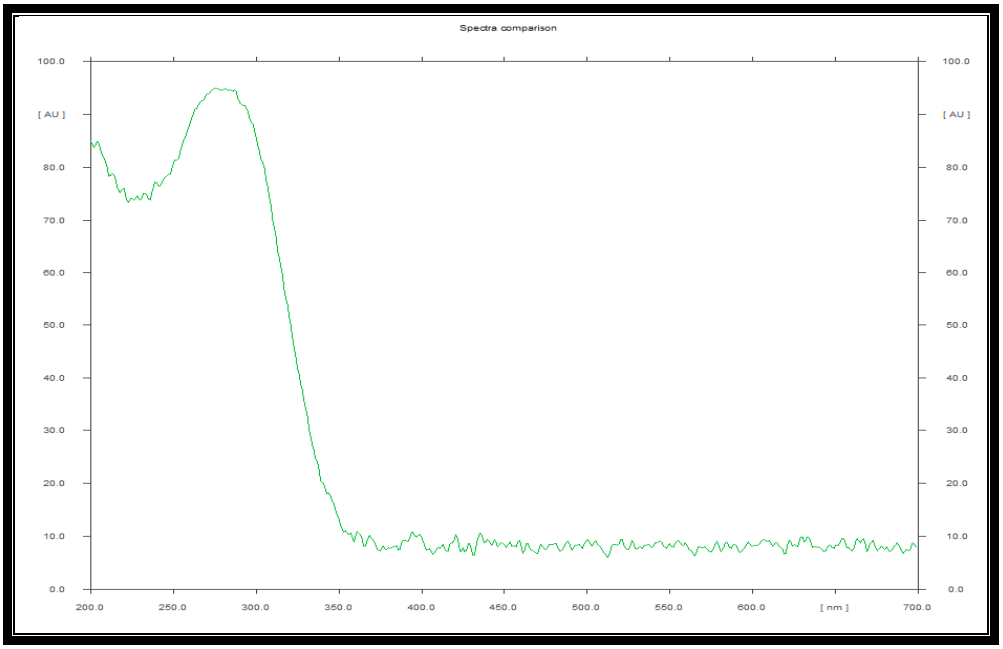


Fig.14 Densitogram of cefpodoxime (100ng/band)

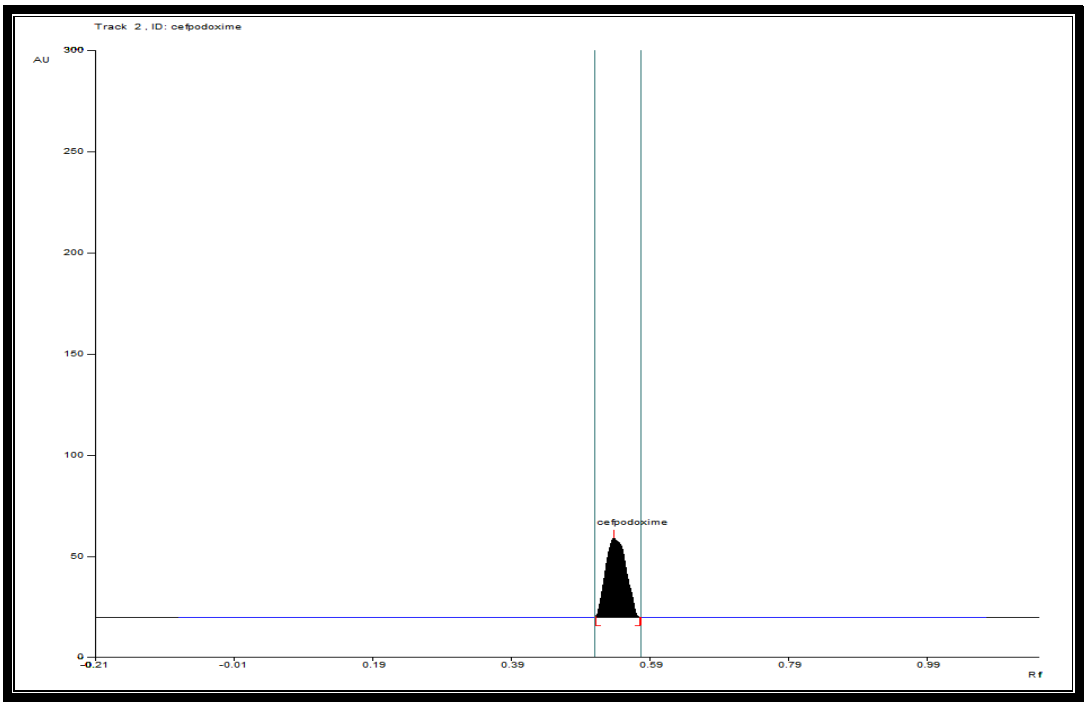


Fig.15 Densitogram of cefpodoxime (150ng/band)

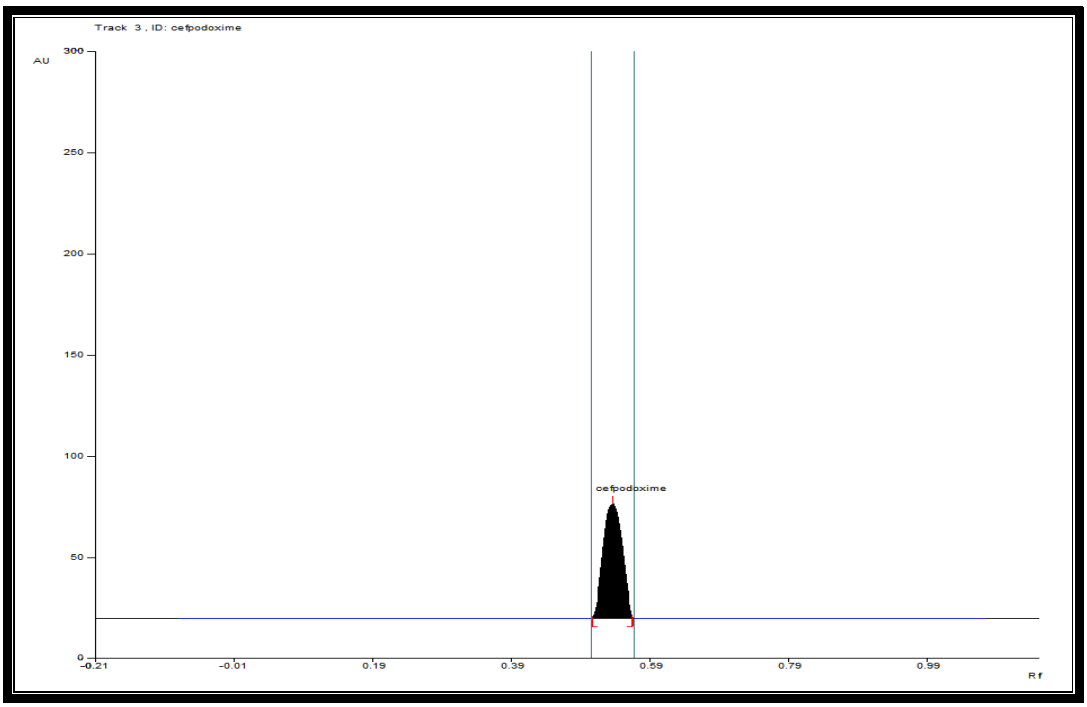


Fig. 16 Densitogram of cefpodoxime (200ng/band)

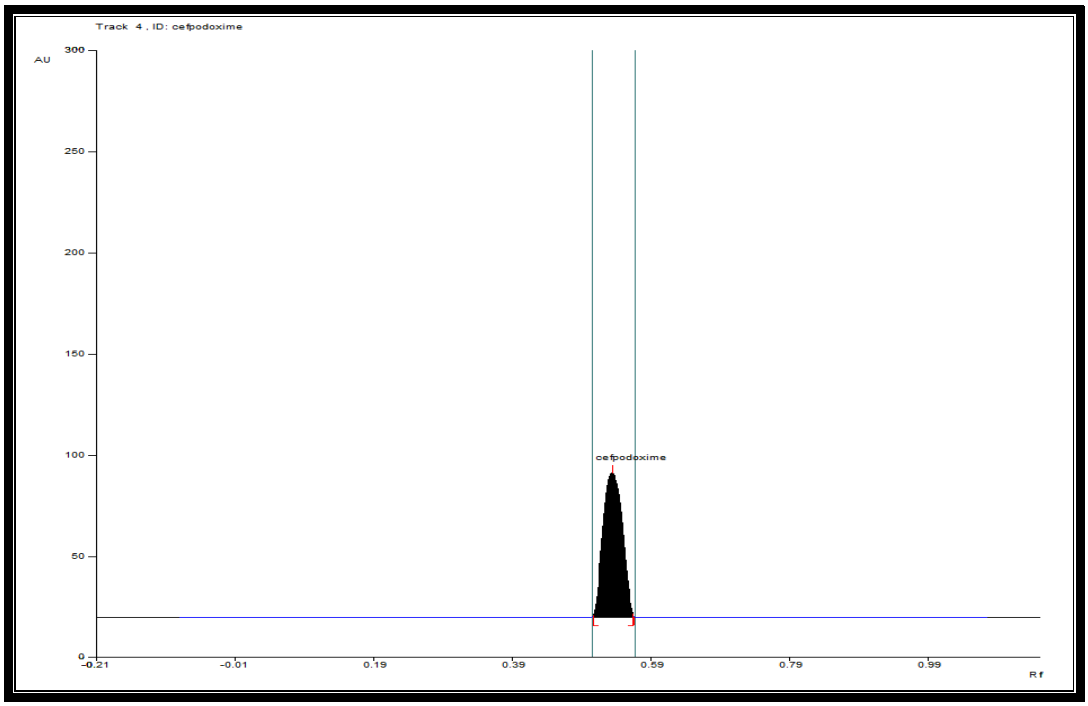


Fig.17 Densitogram of cefpodoxime (250ng/band)

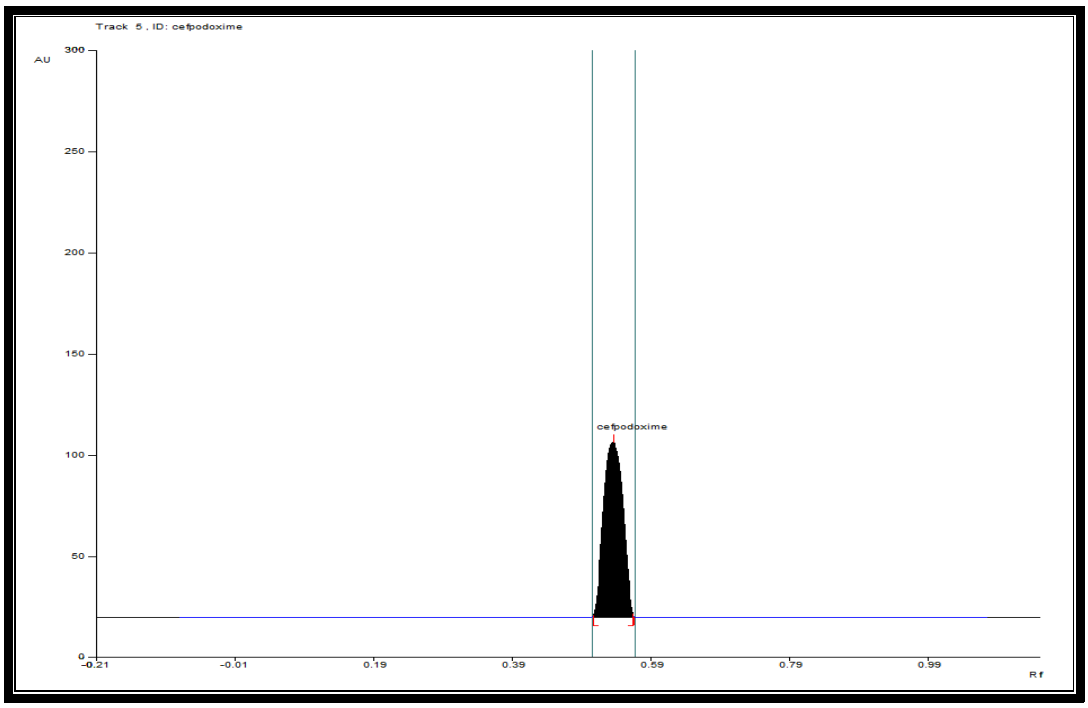
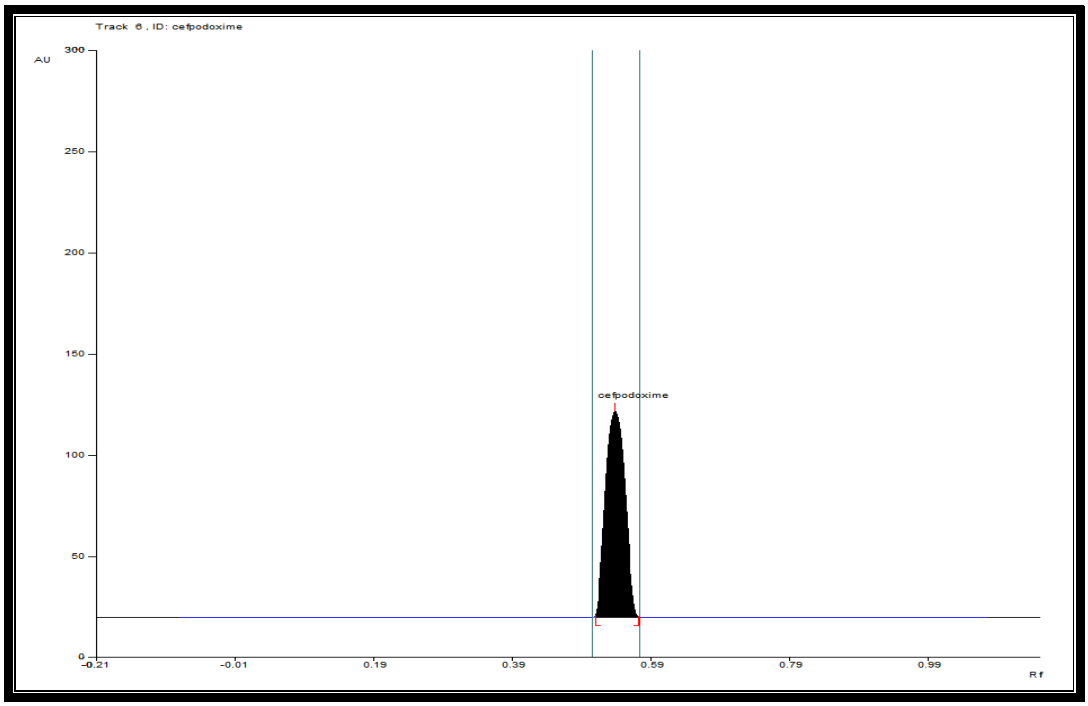


Fig.18 Densitogram of cefpodoxime (300ng/band)



Precision:

The method precision was obtained by repeating the determination of standard drug of two selected concentrations (200 & 250ng/band) of cefpodoxime. The % RSD was calculated for inter-day, intra-day and repeatability (repeatability of sample measurement and sample application respectively) shown in table 10 & 11.

Table.10 Intraday and interday precision

Concentration (ng/band)	Peak area		%RSD	
	Intraday	Interday	Intraday	Interday
200	1817.7	1800.3	1.6	1.2
	1878.6	1845.9		
	1838.6	1820.5		
250	2283.7	2169.8	1.4	1.6
	2178.9	2132.8		
	2135.8	2198.7		

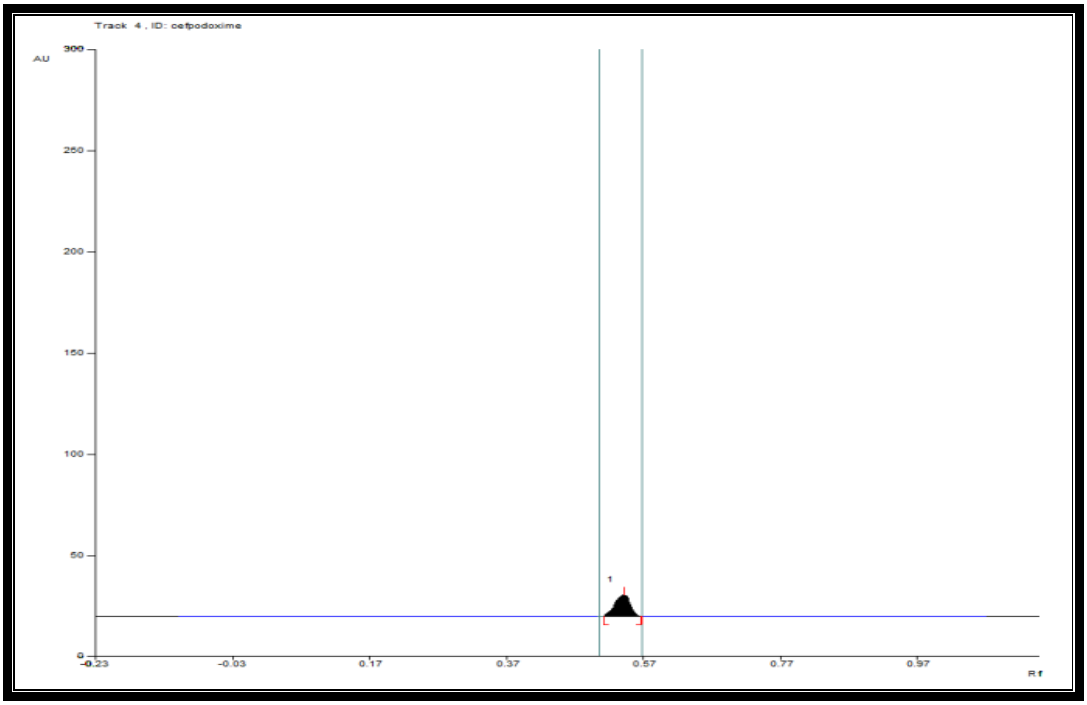
Table 11: Repeatability sample measurement and sample application

Concentration (ng/band)	Peak area		%RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
200	1817.7	2133.2	1.4	1.7
	1878.6	2178.0		
	1830.1	2135.8		
	1856.0	2183.7		
	1838.6	2103.8		
	1878.6	2135.8		

Limit of detection (LOD) and limit of quantitation(LOQ):

The lowest concentration of the analyte detectable was found to be 60ng/band (fig. 19). The lowest concentration of the analyte at which it is quantifiable was found to be 100ng/band.

Fig.19 LOD of cefpodoxime (60ng/band)



Stability of chromatographic plate:

Stability of the analyte on the plate was studied at different time intervals and peak area were compared with the peak area of freshly developed and scanned plate.

Stability of the plate was studied at different time intervals and peak areas were compared with peak area of freshly scanned plate. The developed plate was found to be stable for less than 24hrs (table 12).

Table. 12 plate stability

Time (hr)	Concentration(ng/band)	Peak area
0	100 250	1230.4 1894.6
8	100 200	1083.9 1640.6

ANALYSIS OF FORMULATION:

Preparation of sample solution for cefpodoxime:

Each of twenty tablets containing 200mg of cefpodoxime was taken for the study and average weight was determined. Quantity equivalent to 10mg cefpodoxime was weighed and transferred to 100ml standard flask and after dissolving it was made up to volume with methanol. It was filtered and used for analysis.

Recording chromatogram:

The fixed chromatographic condition, a suitable volume of sample solution was applied on the precoated TLC plate. The plate was analyzed and chromatogram as recorded. The amount present per tablet, % label claim were calculated and shown in table 13. The UV spectrum of spot is shown in fig. 20 and chromatogram of formulation is shown in fig 21.

Table. 13 Results of analysis of formulation

Brand	Amount found		% Label claim	% RSD
	Label mg/tablet	Found mg/tablet		
BALPOD	200	195.2	97.6%	0.96

Fig. 20 Spectrum of cefpodoxime band

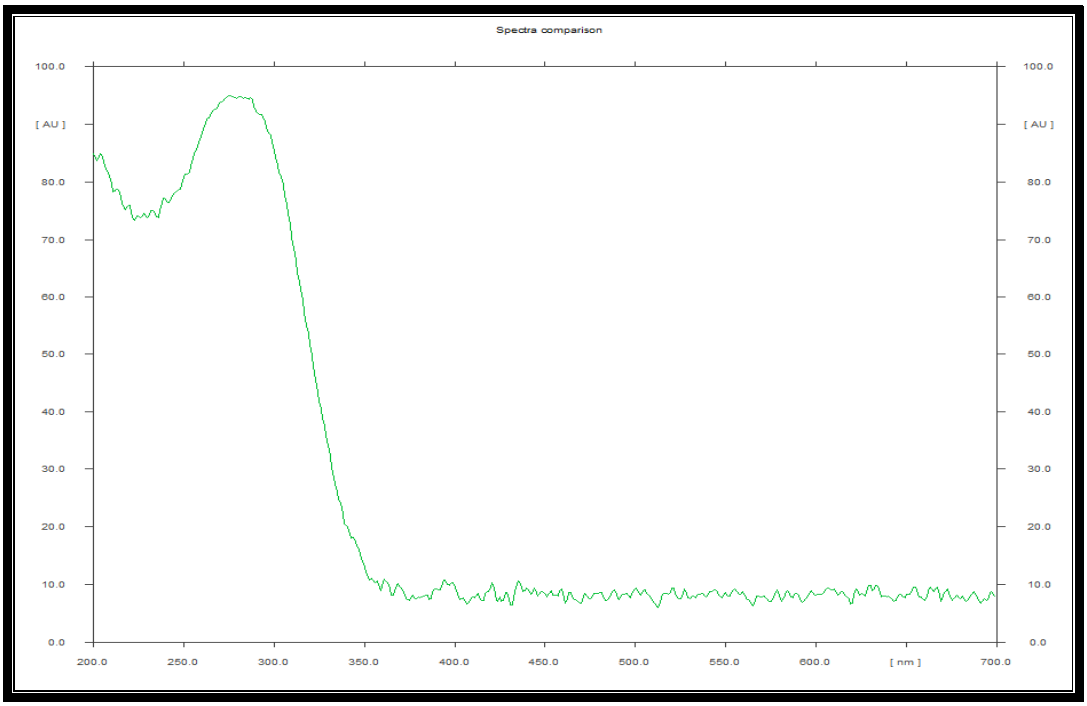
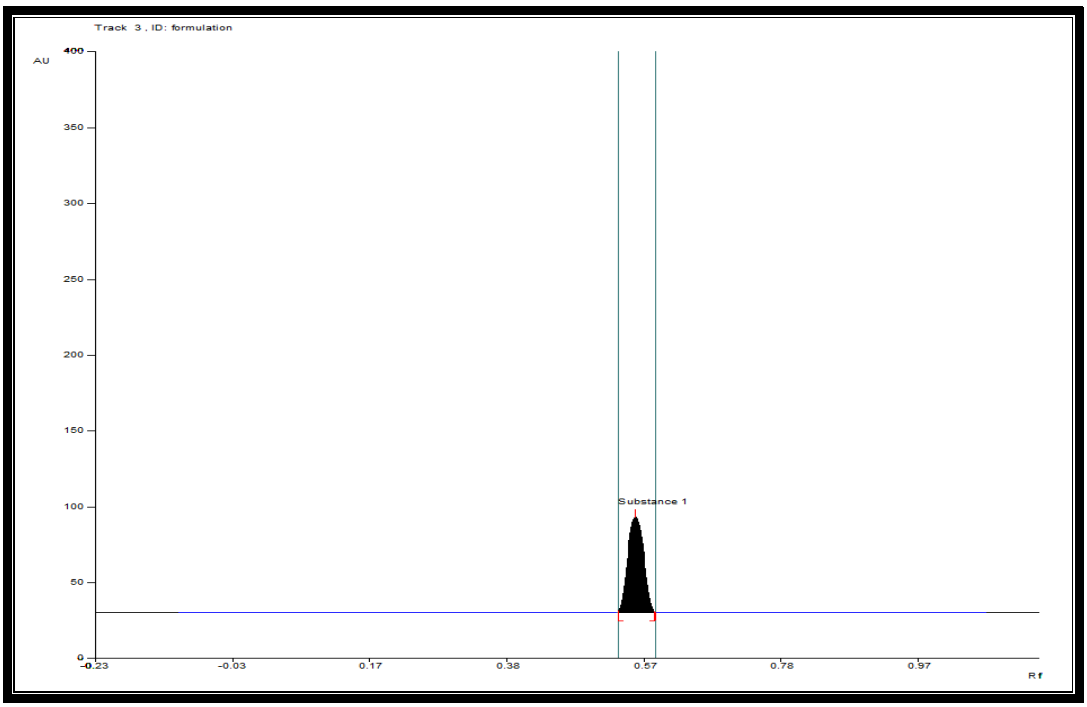


Fig.21 Densitogram of cefpodoxime formulation



Recovery:

Recovery studies were carried out at 80% and 100% levels. The percentage recovery and percentage RSD of the results were calculated and shown in table 14. The value proves the accuracy of the method.

Table.14 Recovery studies

Levels	% Recovery	%RSD*
80	98.55%	0.51
100	101.4%	0.82

*Average of six observation

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR DETERMINATION OF CEFEPIME IN BULK AND PHARMACEUTICAL DOSAGE FORM

Among various mobile phase system tried the one contain a mixture of methanol: water: chloroform (6: 3: 1 % v/v/v) gave compact dense band was chosen for the estimation of benfotiamine by HPTLC method. The plate used was pre-coated silica gel $G_{60}F_{254}$. The method was for the validated and details given below.

Linearity and range:

A 100µg/ml of cefixime was prepared in methanol. Aliquots of this solution (5-25µl) were applied on the plate. The plate was developed, scanned and peak areas were noted. Linear regression data showed good correlation coefficient over a concentration range of (500-2500ng/band). The calibration graphs are shown in fig 22. The slope, intercept, and correlation coefficient values were in noted and showen in table 15. The spectra of spot recorded on HPTLC scanner (fig 23) and the standard densitograms are shown in fig.24 to 28 and calibration data were shown table 16.

Table: 15 Regression data of cefepime

Linear regression	cefepime
Slope	1.2
Intercept	85.36
Correlation coefficient	0.998

Fig.22 calibration graph for cefepime

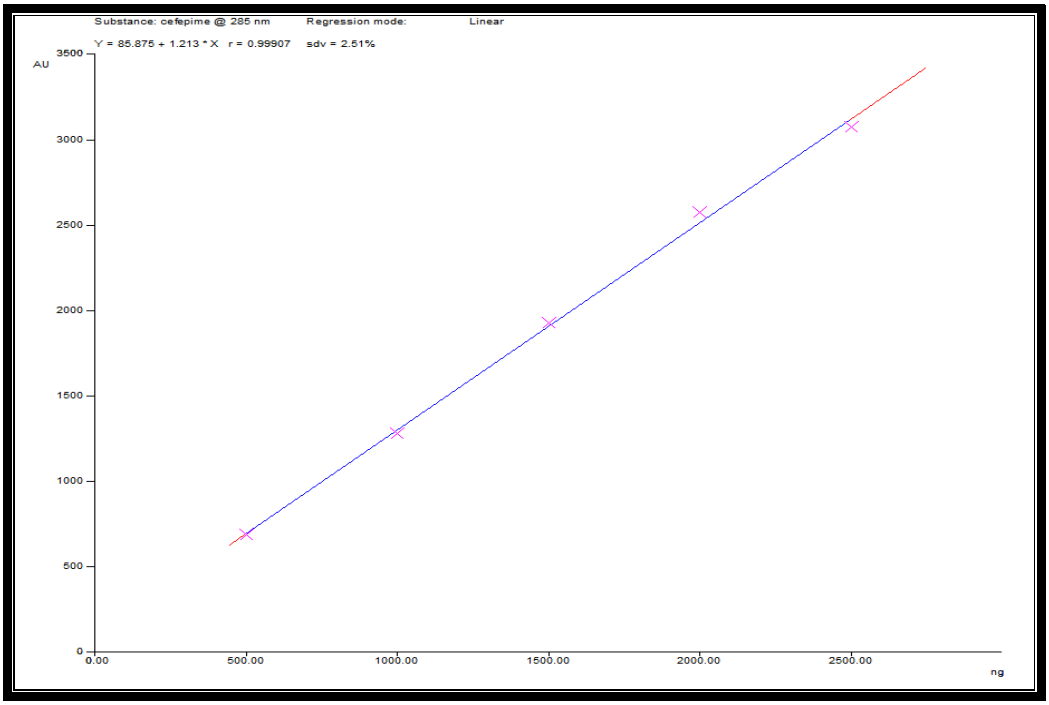


Fig.23 Spectrum of cefepime band

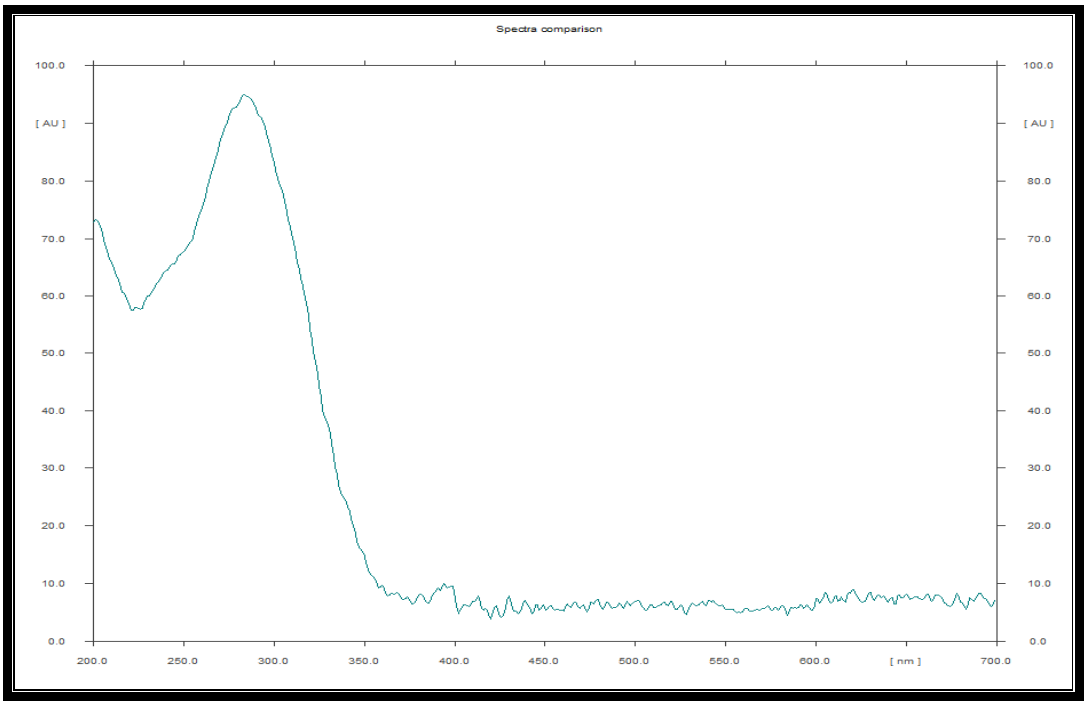


Table: 16 calibration data of cefepime

Concentration(ng/band)	Peak area
500	668.1
1000	1348.8
1500	1939.6
2000	2516.5
2500	3198.5

Fig.24 Densitogram of cefepime 500ng/band

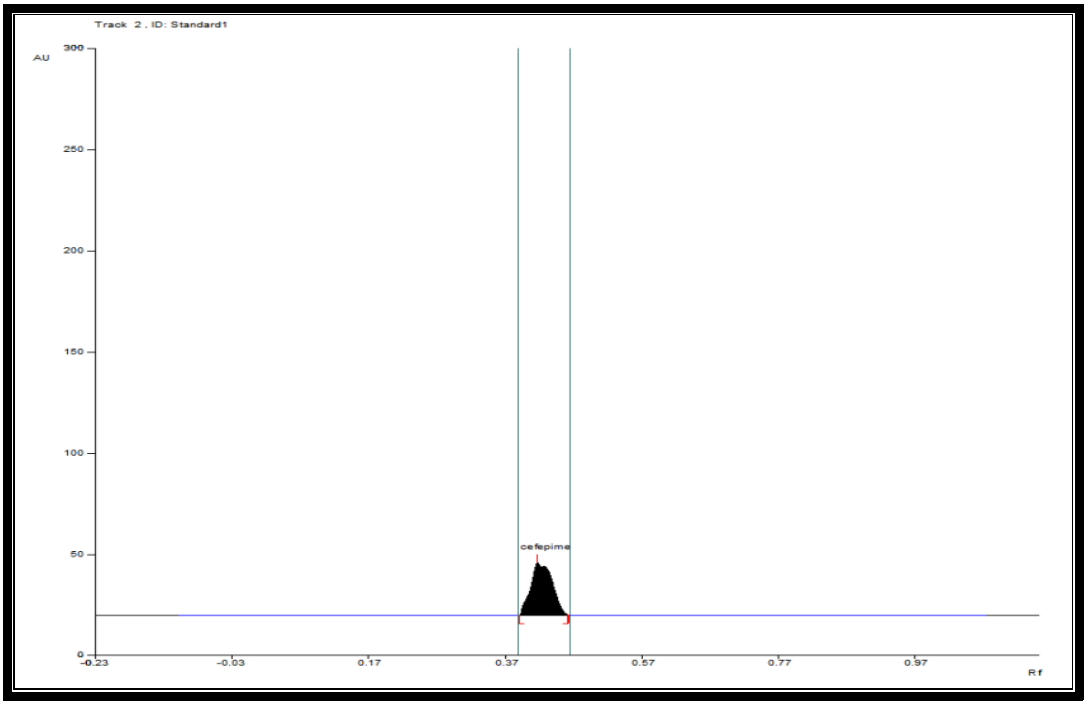


Fig.25 Densitogram of cefepime 1000ng/band

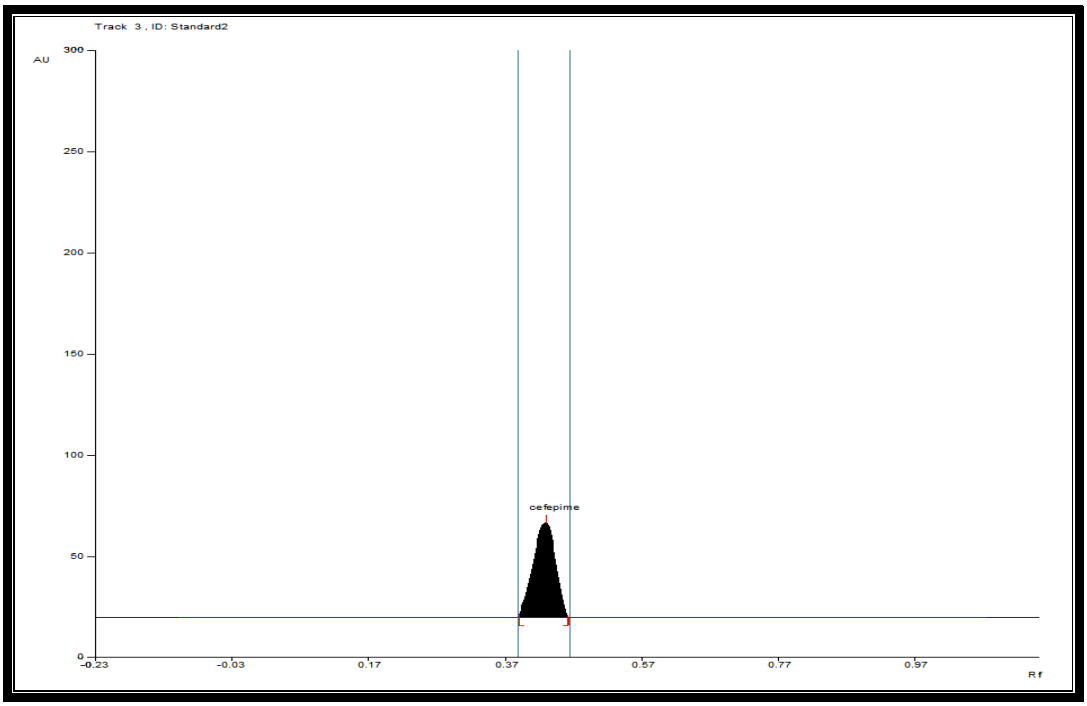


Fig.26 Densitogram of cefepime 1500ng/band

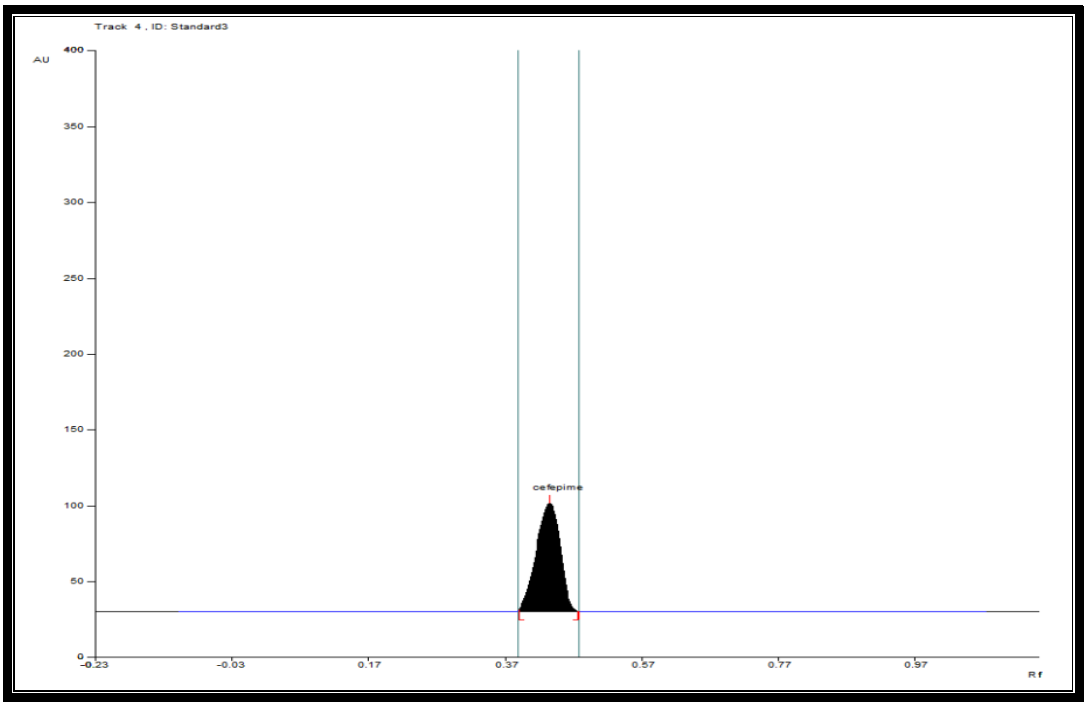


Fig.27 Densitogram of cefepime 2000ng/band

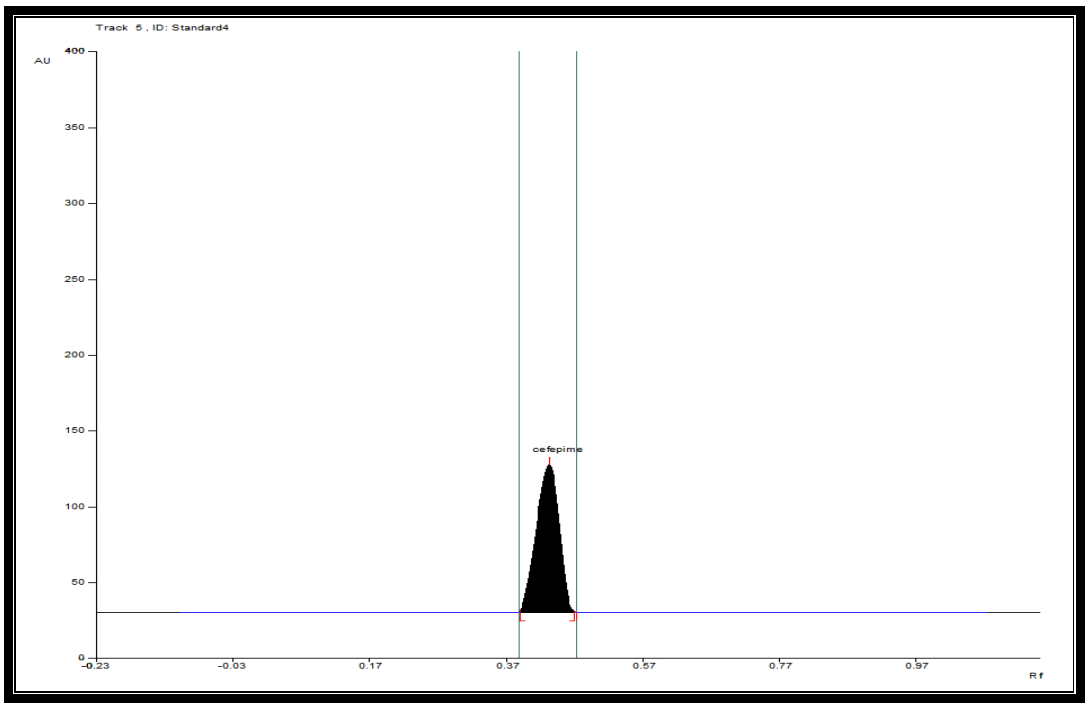
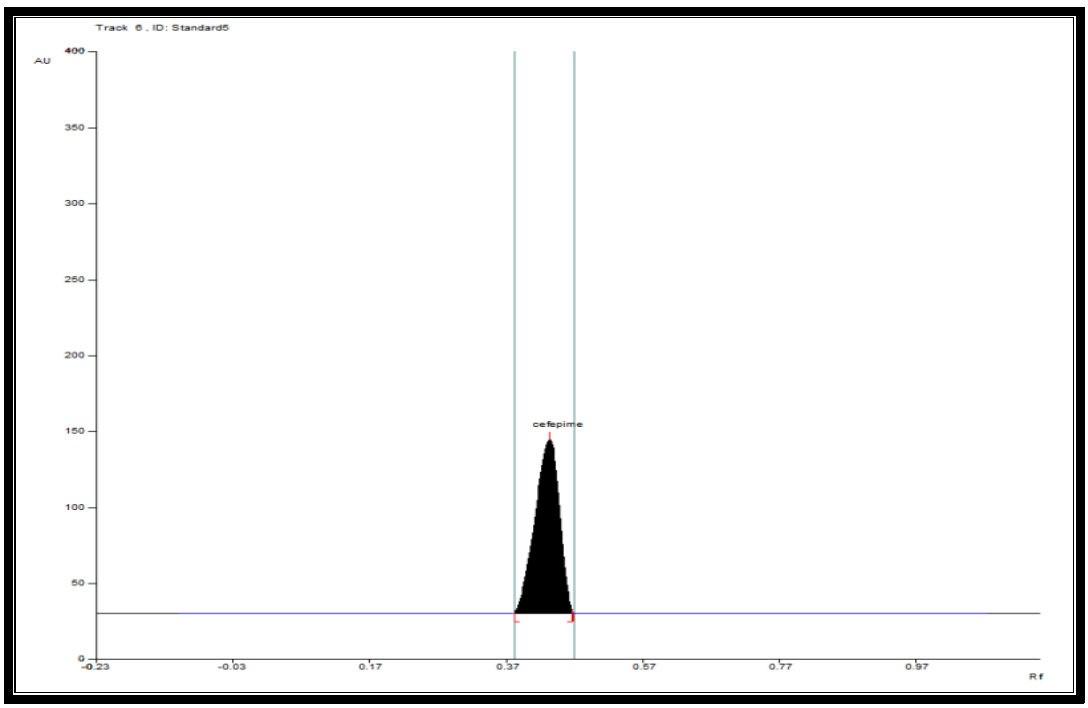


Fig.28 Densitogram of cefepime 2500ng/band



Precision:

The method precision was obtained by repeating the determination of standard drug of two selected concentrations (1500 & 2000ng/band) of cefepime. The % RSD was calculated for inter-day, intra-day and repeatability (repeatability of sample measurement and sample application respectively) shown in table 17 & 18.

Table: 17 Intraday and interday precision

Concentration(ng/band)	Peak area		%RSD	
	Intraday	Interday	Intraday	Interday
1500	1866.0	1808.5	0.8	0.6
	1888.7	1832.8		
	1898.0	1823.3		
2000	2599.0	2513.9	0.3	0.4
	2577.7	2514.9		
	2589.0	2533.2		

Table: 18 Repeatability sample measurement and sample application

Concentration (ng/band)	Peak area		%RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
2000	1779.0	1755.9	1.1	1.2
	1755.8	1733.6		
	1723.4	1799.5		
	1763.8	1751.8		
	1745.8	1763.8		
	1777.1	1763.8		

Limit of detection (LOD) and limit of quantitation(LOQ):

The lowest concentration of the analyte detectable was found to be 120ng/band. The lowest concentration of the analyte at which it is quantifiable was found to be 250ng/band

Stability of chromatographic plate:

When the developed method chromatographic plate was exposed to atmosphere, the analytes are likely to decomposed. Hence it was necessary to conduct stability of the plate.

Stability of the plate was studied at different time intervals and peak areas were compared with peak area of freshly scanned plate. The developed plate was found to be stable for less than 24hrs (table 19).

Table. 19 plate stability

Time (hr)	Concentration(ng/band)	Peak area
0	1000 1500	1348.8 1939.6
8	1000 1500	810.0 1081.1

ANALYSIS OF FORMULATION:

Preparation of sample solution for cefepime:

Ten formulation of Powder for injection (1g/10ml) of cefepime was taken for the study and. Quantity equivalent to 10mg cefepime was weighed and transferred to 100ml standard flask and after dissolving it was made up to volume with methanol. It was filtered and used for analysis.

Recording chromatogram:

The fixed chromatographic condition, a suitable volume of sample solution was applied on the precoated TLC plate. The plate was analyzed and chromatogram as recorded. The amount present per tablet, % label claim were calculated and shown in table 20. The UV spectrum of spot is shown in fig 29 and chromatogram is presented in fig 30.

Fig. 29 Densitogram of cefepime

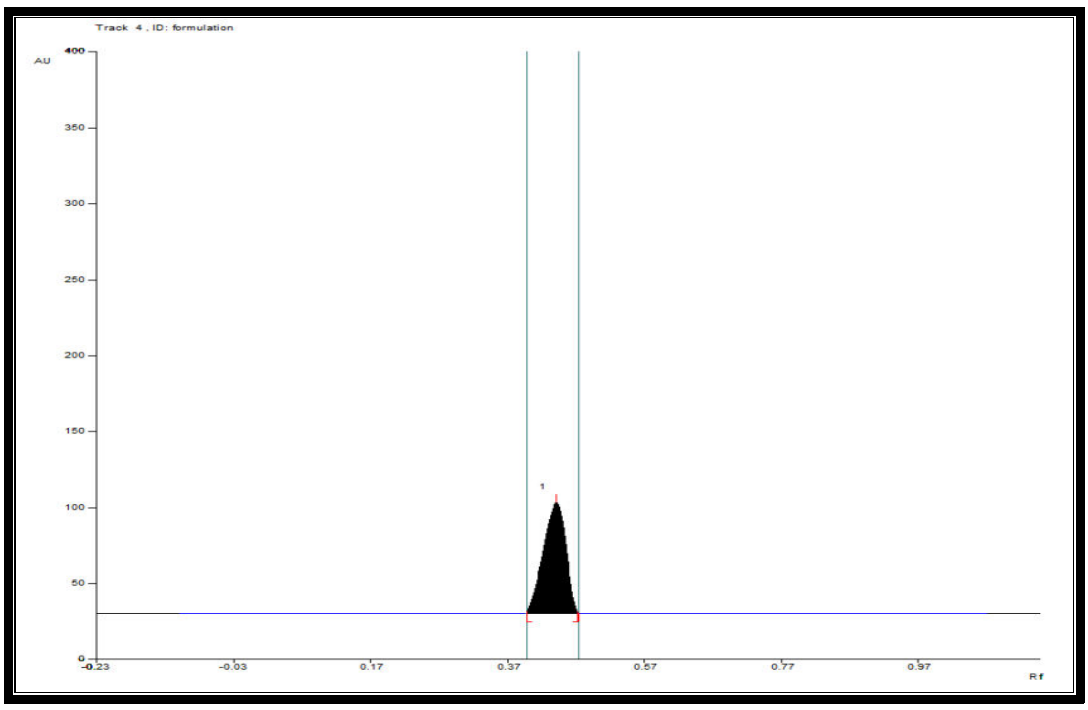


Fig.30 spectrum of cefepimeband

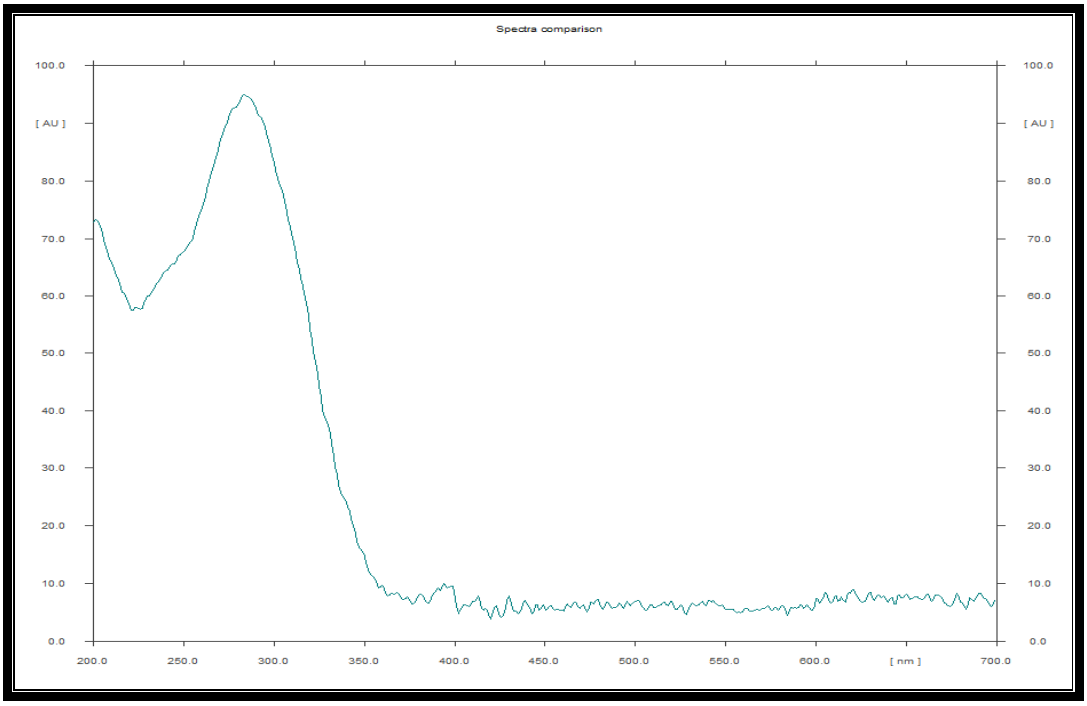


Table.20 Results of analysis of formulation

Brand	Amount found		% Label claim	% RSD
	Label 1000mg/ml	Found 1000mg/ml		
NOVAPIME	1000	997.2	99.7%	0.37

Recovery:

Recovery studies were carried out at 80% and 100% levels. The percentage recovery and percentage RSD of the results were calculated and shown in table 14. The value proves the accuracy of the method.

Table.21 Recovery studies

Levels	% Recovery	%RSD*
80	97.9%	0.27
100	99.8%	0.54

*Average of six observation

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR ESTIMATION OF BRIVUDINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

Different mobile phase compositions are tried for development and validation of brivudine by HPTLC method. Among these mobile phase system, methanol: chloroform: toluene (2:5:3 % v/v) was selected because in this system compact and dense band with good separation were obtained.

Linearity and range:

Linear regression data showed a good correlation coefficient over a concentration range 50-300ng/band. The calibration data are shown in the (table 22) and the slope, intercept and correlation coefficient values were found are presented in (table 23) respectively. The calibration graph is shown in fig 31. The spectrum recorded on HPTLC scanner (fig 32) and the standard chromatograms obtained at different concentrations of brivudine are shown in fig. 33-38.

Table 22: Calibration data for brivudine

Concentration (ng/band)	Peak Area
50	1389.0
100	2642.8
150	3705.8
200	473101
250	5578.9
300	6459.5

Table 23: Regression data for brivudine

Slope	20.106
Intercept	565.907
Correlation coefficient	0.997

Fig. 31 calibration graph of brivudine:

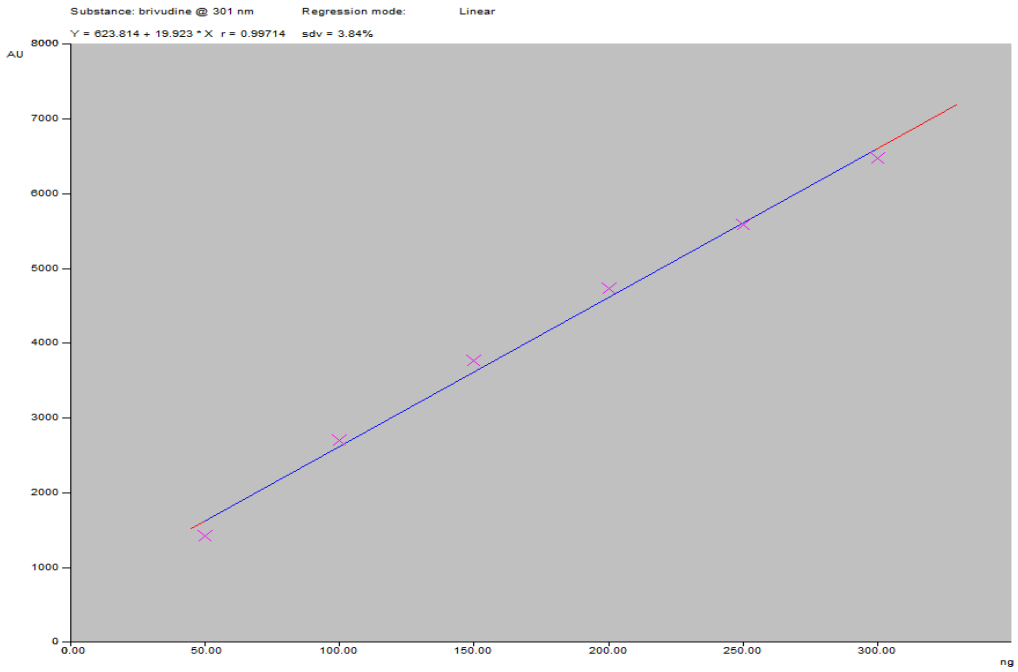


Fig. 32 spectrum of brivudine band

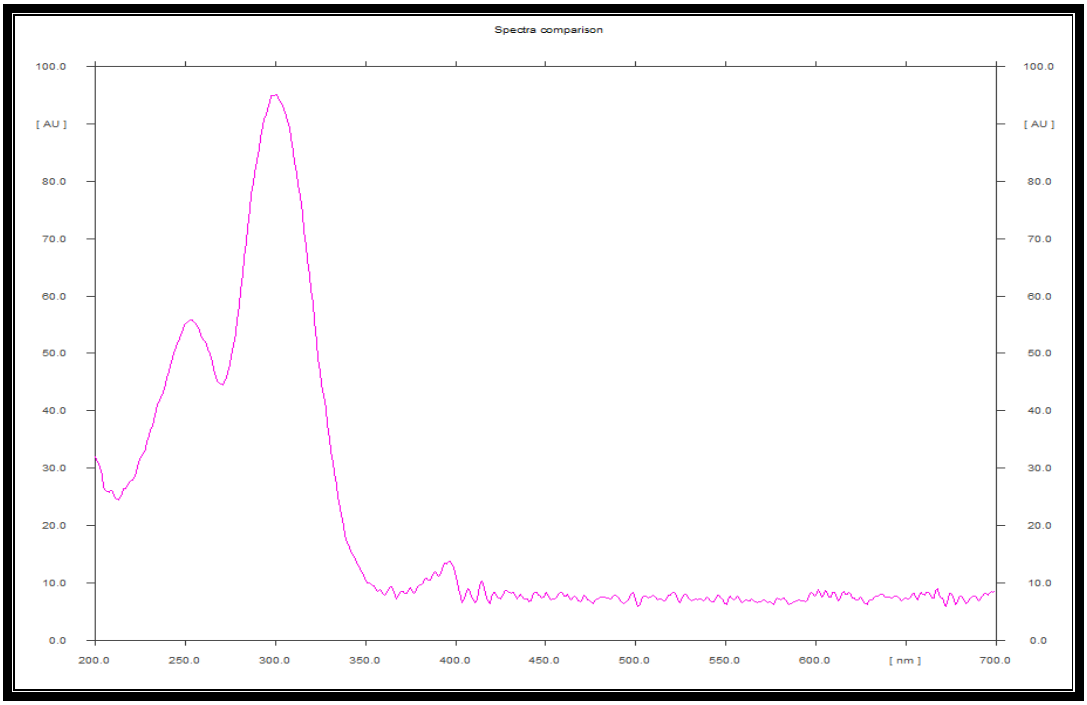


Fig. 33 Densitogram of brivudine (50ng/band)

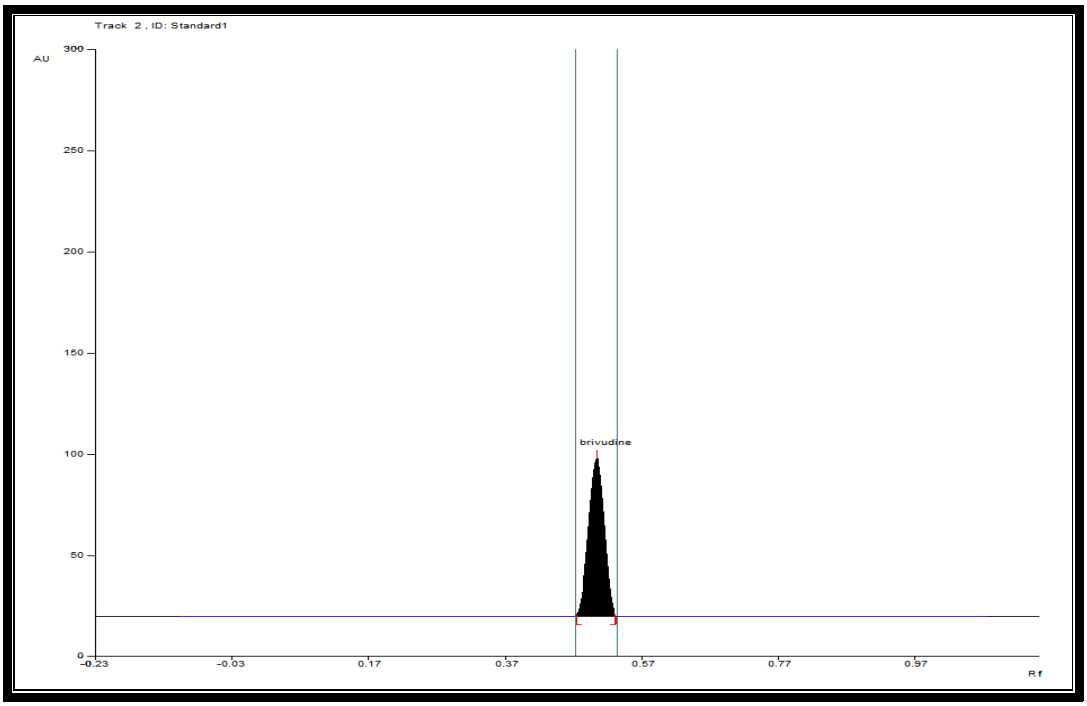


Fig. 34 Densitogram of brivudine (100ng/band)

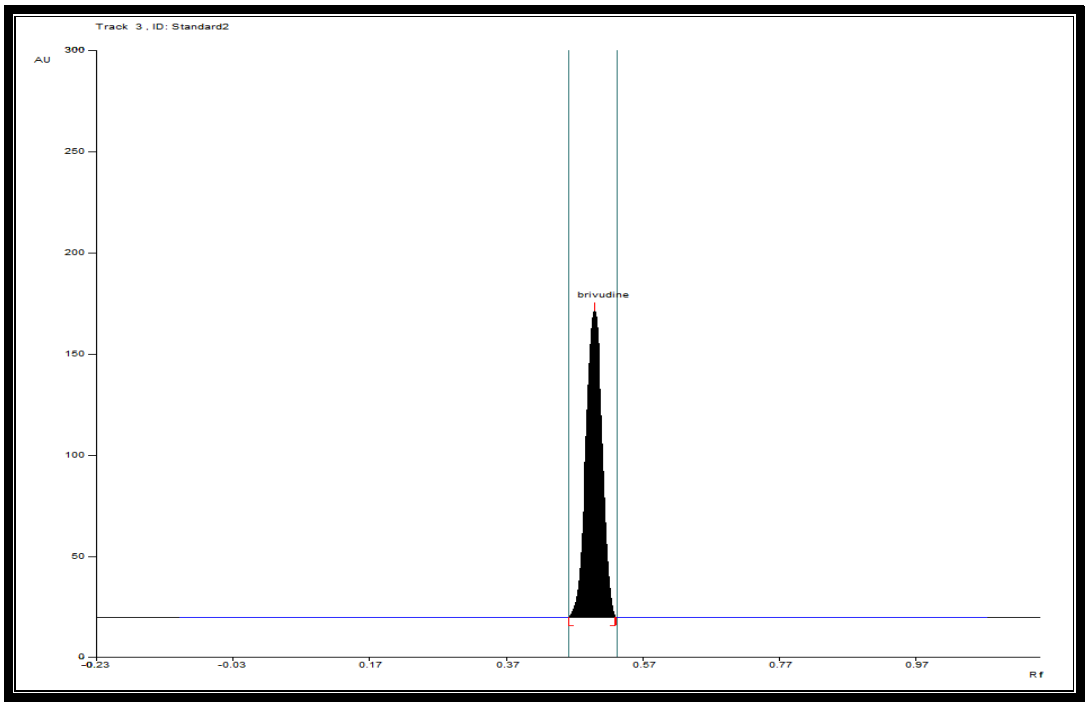


Fig. 35 Densitogram of brivudine (150ng/band)

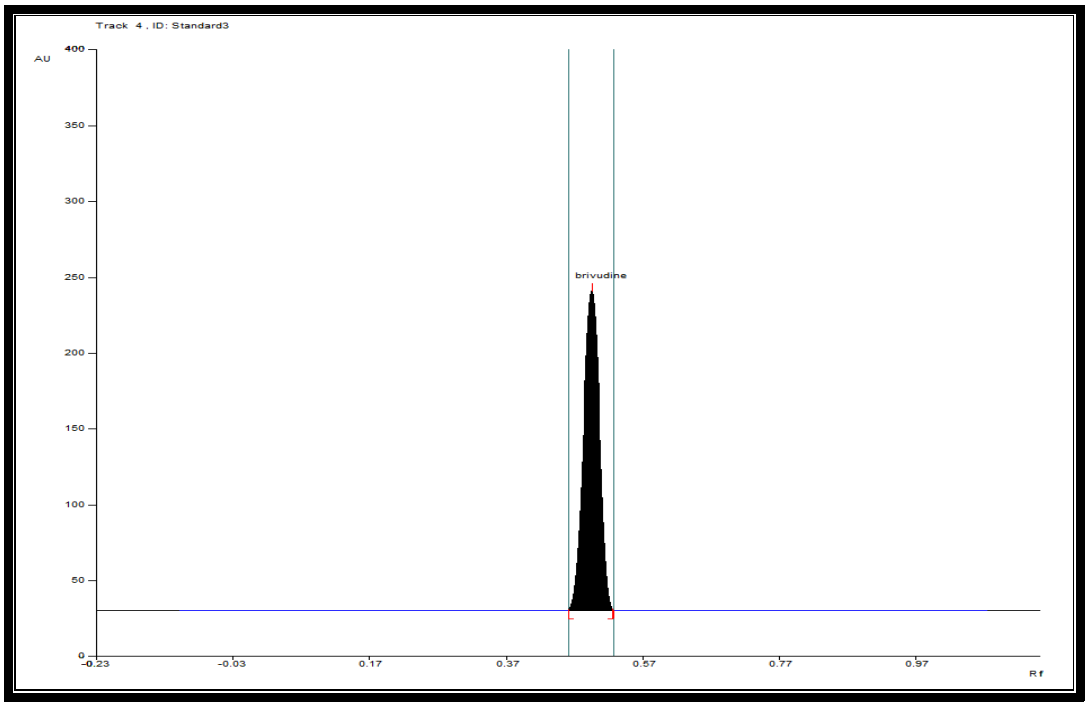


Fig. 36 Densitogram of brivudine (200ng/band)

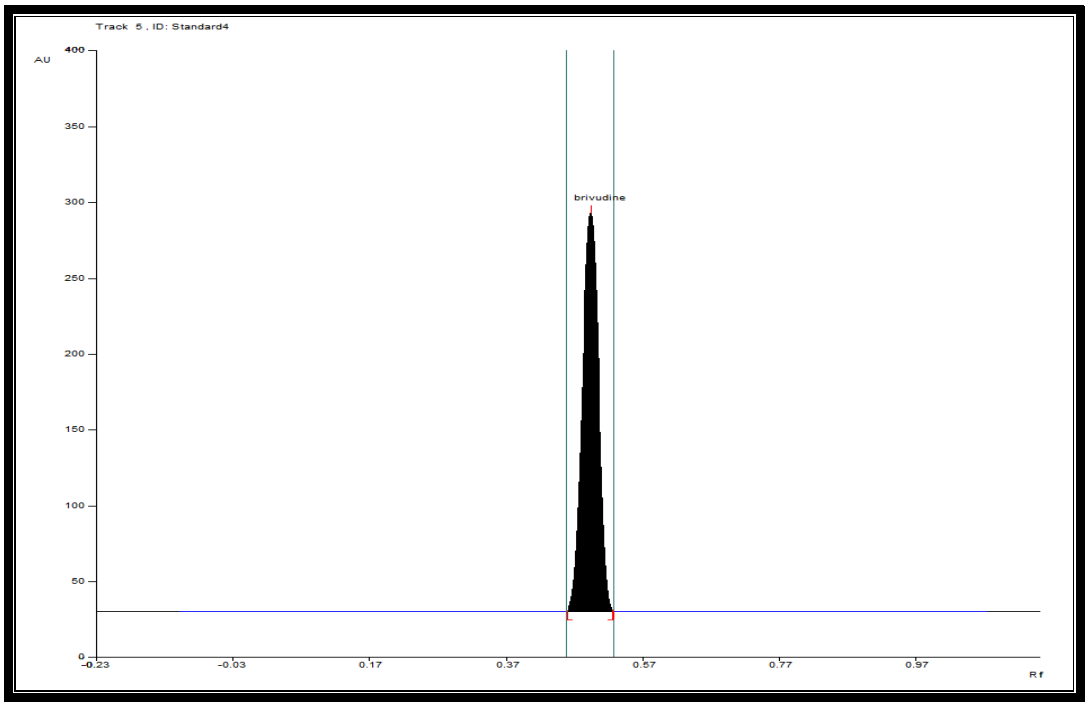


Fig. 37 Densitogram of brivudine (250ng/band)

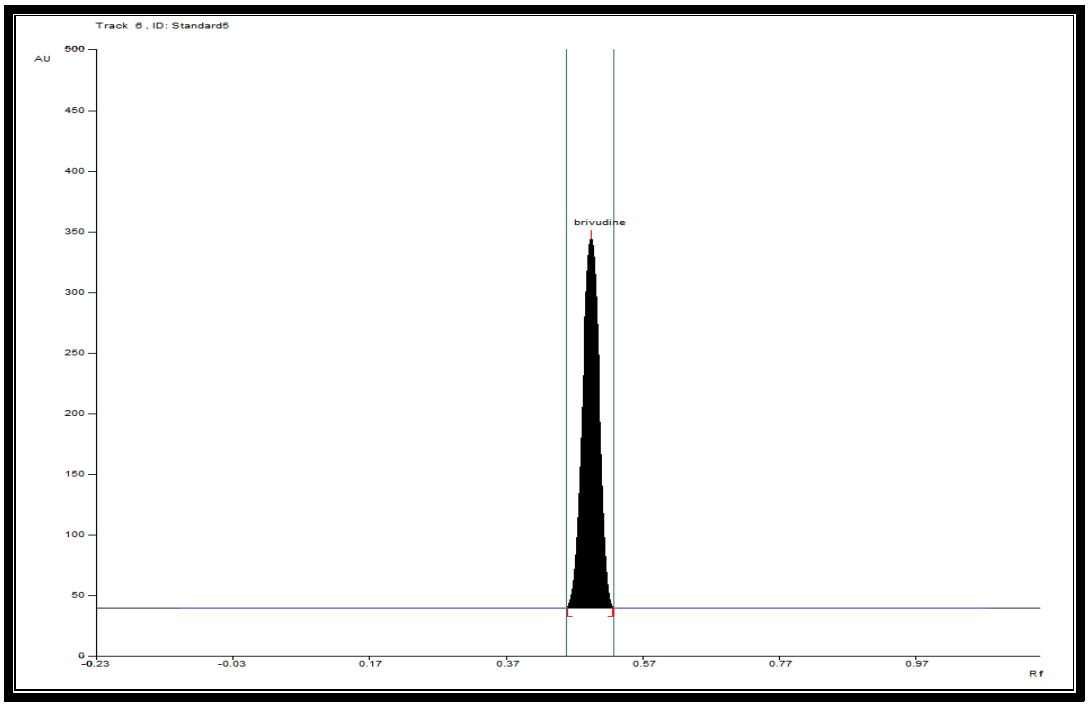
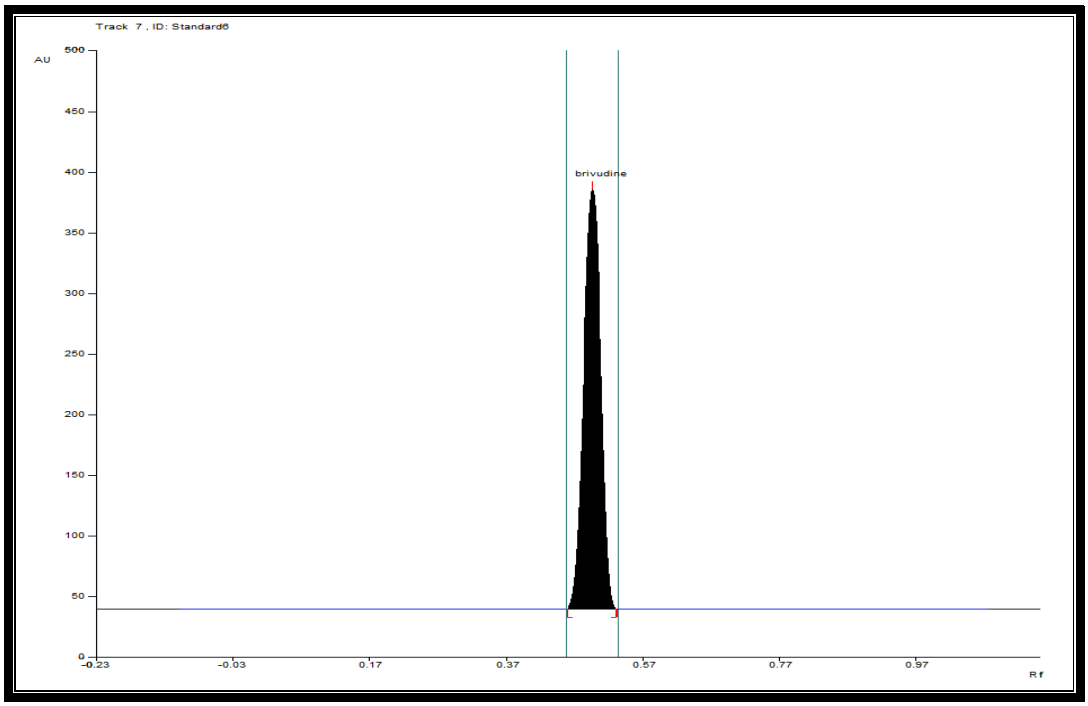


Fig. 38 Densitogram of brivudine (300ng/band)



Precision:

The method precision was obtained by repeating the determination of standard drug of two selected concentrations (150 & 200ng/band) of brivudine. The % RSD was calculated for inter-day, intra-day and repeatability (repeatability of sample measurement and sample application respectively) shown in table 24 &25.

Table.24 Intraday and interday precision

Concentration (ng/band)	Peak area		%RSD	
	Intraday	Inter day	Intraday	Inter day
150	3770.4	3662.6	0.9	0.6
	3735.6	3657.9		
	3772.1	3690.5		
200	4798.8	4663.1	0.2	1.4
	4744.5	4602.3		
	4733.4	4763.1		

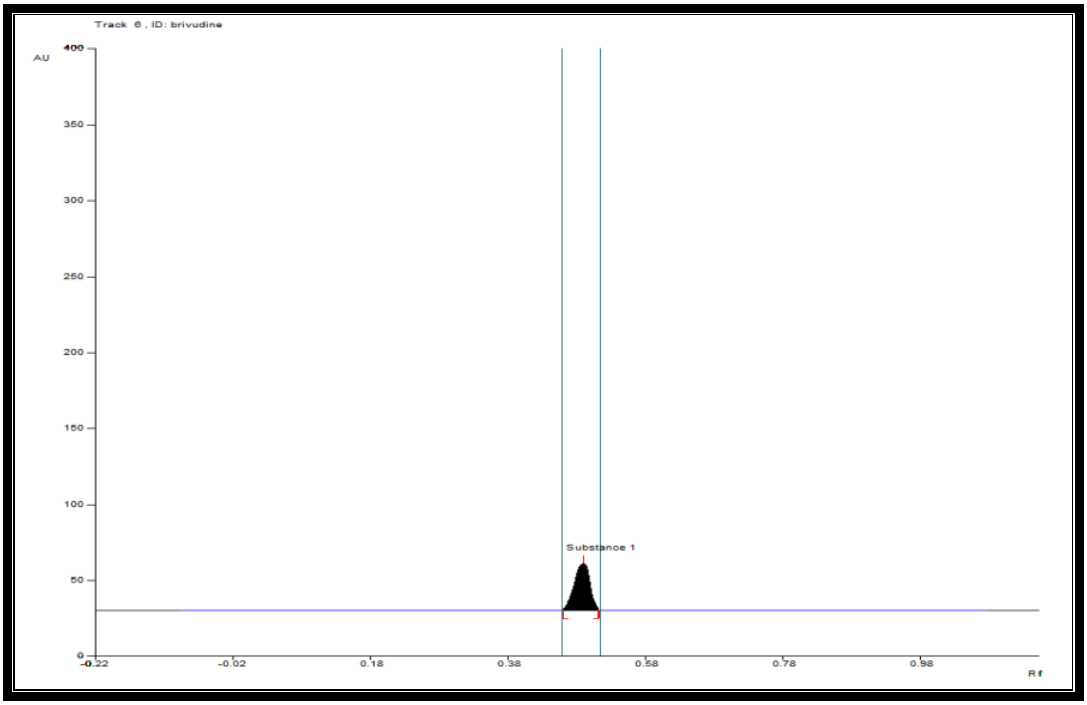
Table. 25 Repeatability sample measurement and sample application

Concentration (ng/band)	Peak area		%RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
200	4733.6	4732.6	0.6	0.5
	4789.5	4769.8		
	4786.4	4702.8		
	4723.8	4710.3		
	4754.9	4728.8		
	4712.3	4700.3		

Limit of detection (LOD) and limit of quantitation(LOQ):

The lowest concentration of the analyte detectable was found to be 30ng/band (fig. 39). The lowest concentration of the analyte at which it is quantifiable was found to be 50ng/band.

Fig . 39 LOD of brivudine (30ng/band)



Robustness:

The robustness of the method was proven by deliberate changes in condition like mobile phase ratio (±0.5ml) and staturation time (± 3min). the Rf values and peak area were found to be similar hence method is said to be robust.

Stability of chromatographic plate:

When the developed method chromatographic plate was exposed to atmosphere, the analytes are likely to decomposed. Hence it was necessary to conduct stability of the plate.

Stability of the plate was studied at different time intervals and peak areas were compared with peak area of freshly scanned plate. The developed plate was found to be stable for less than 24hrs,(table 26).

Table 26: plate stability

Time (hr)	Concentration(ng/band)	Peak area
0	250 300	5578.9 6459.5
8	250 300	5499.0 6400.1
24	250 300	4268.5 4676.5

ANALYSIS OF FORMULATION:

Preparation of sample solution for Brivudine:

Each of twenty tablets containing 125mg of brivudine was taken for the study and average weight was determined. Quantity equivalent to 10mg brivudine was weighed and transferred to 10ml standard flask and after dissolving it was made up to volume with methanol. It was filtered and used for analysis.

Recording chromatogram:

The fixed chromatographic condition, a suitable volume of sample solution was applied on the precoated TLC plate. The plate was analyzed and chromatogram as recorded. The amount present per tablet, % label claim were calculated and shown in table 27. The UV spectrum of brivudine is shown in fig 40 and chromatogram of spot is shown in fig 41.

Fig.40 spectrum of brivudine band

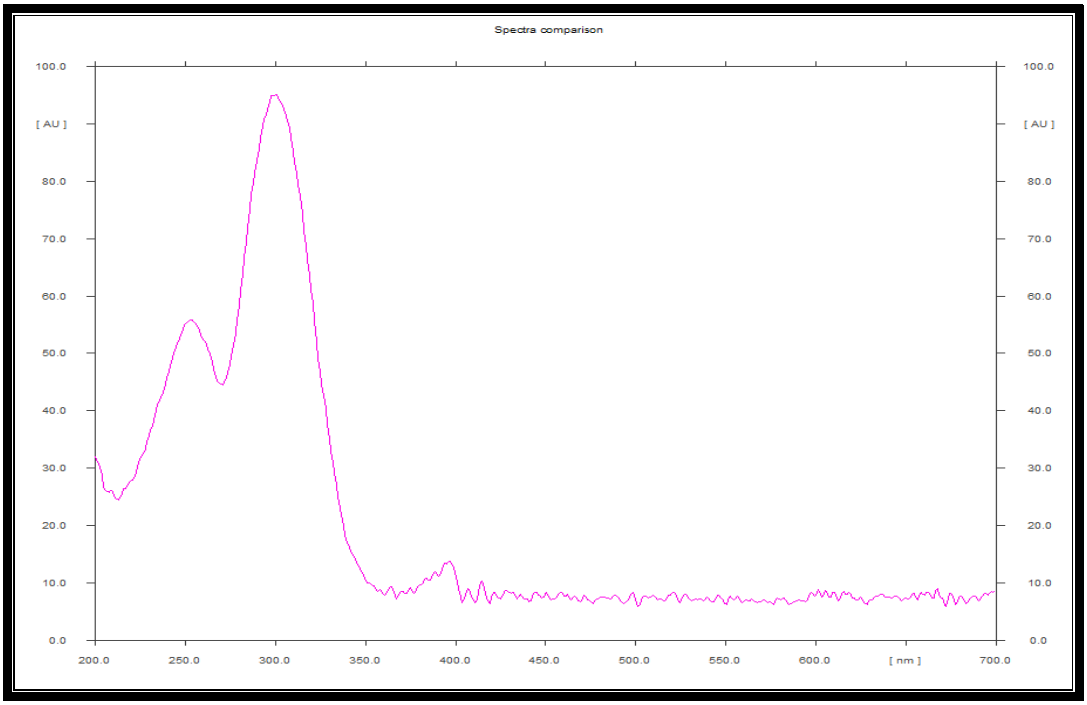


Fig.41 Densitogram of brivudine (100ng/band)

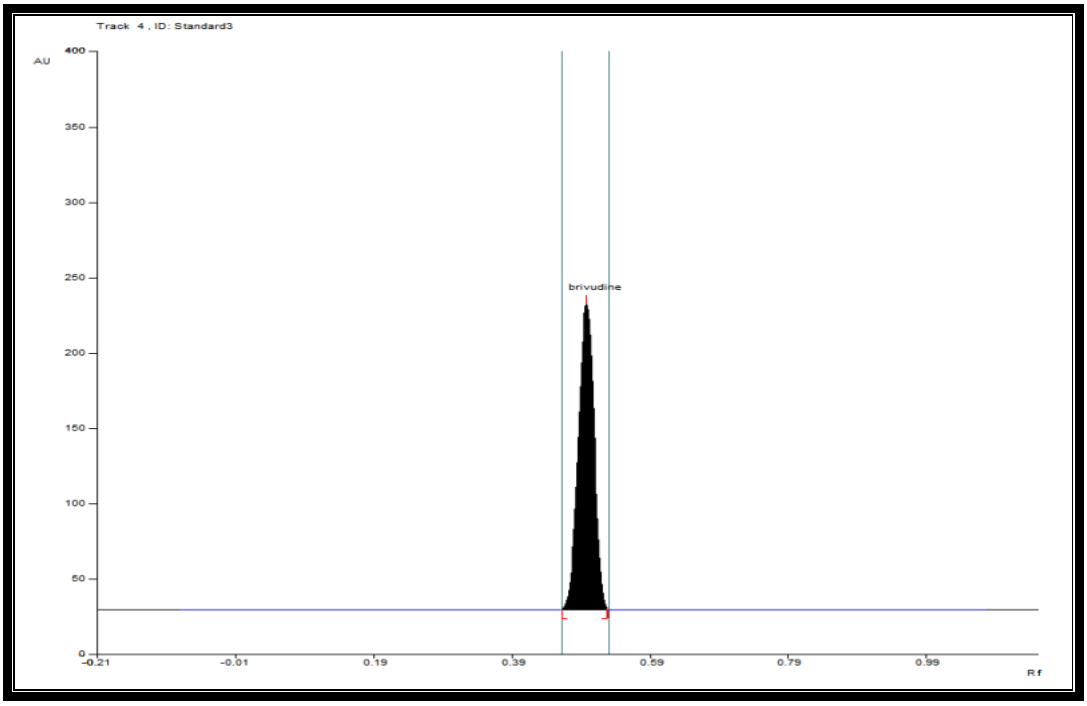


Table 27: results of analysis of formulation:

Brand name	Amount of drug/tablet		%label claim	%RSD*
	Labeled(mg)	found(mg)		
Zostex	125	122	97%	0.9

***Average of six observation**

Recovery:

Recovery studies were carried out at 80% and 100% levels. The percentage recovery and percentage RSD of the results were calculated and shown in table 28. The value proves the accuracy of the method.

Table 28: recovery studies:

Level	% Recovery	% RSD*
80%	101.07%	0.32
100%	100.9%	0.29

***Average of six observation**

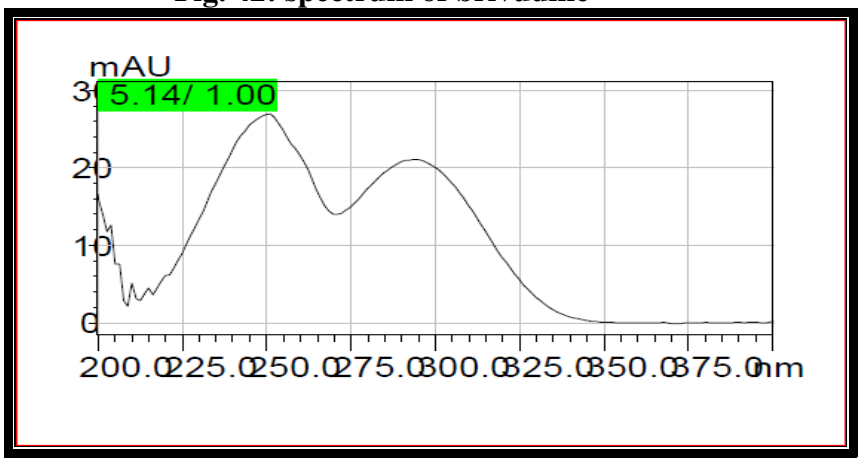
DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF BRIVUDINE IN BULK AND PHARMACEUTICAL DOSAGE FORM

Brivudine is a polar in nature hence RP-HPLC method and C18 column was used for the HPLC method development for the estimation of brivudine.

Selection of wavelength:

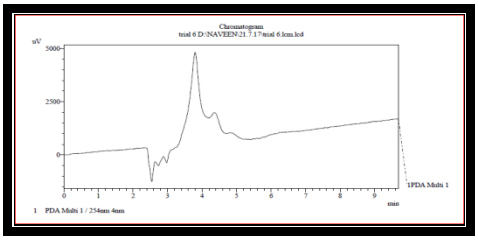
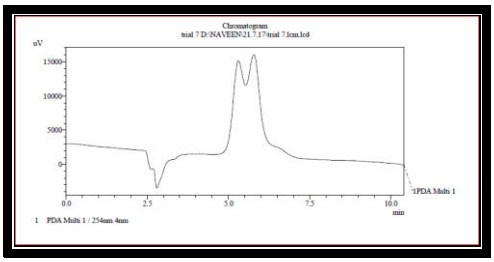
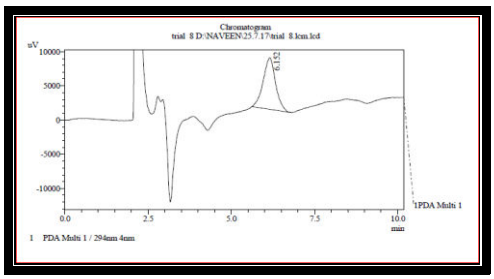
Good analytical separation can be obtained only by careful selection of wavelength for the detection (fig.42). The smooth obtained from which 254nm was select for a drug.

Fig. 42: spectrum of brivudine



Selection of mobile phase:

For developing HPLC method, mobile phase system containing 1% ortho phosphoric acid (pH adjusted with triethyl amine): methanol was tried different ratios and different pH. The observation and chromatograms are given below table 29. A ratio containing 40:60 % v/v and at a pH 6.5 was selected as ideal one.

Mobile phase	Ratio (% v/v)	chromatograms	pH	observation
Buffer: methanol	50:50		3.5	Broad peak
Buffer: methanol	60:40		3.5	Split peak
Buffer: methanol	40:60		6.5	Good peak separation

Linearity and range:

A calibration graph was plotted with measured peak area against concentration. From the graph it was found that brivudine shows good linearity in the concentration between 0.5-3.0mcg/ml. The peak area of these solutions was measured at 254nm. The slope, intercept, and correlation coefficient values were calculated respectively (table 30). The linearity table is shown in table 30. The calibration graph and standard chromatogram were obtained and shown in fig 43-49.

Table 30 Calibration data

Concentration (µg/ml)	Peak Area
0.5	48264
1	78565
105	107762
2	133056
2.5	156059
3	185107

Fig. 43 Calibration graph of brivudine

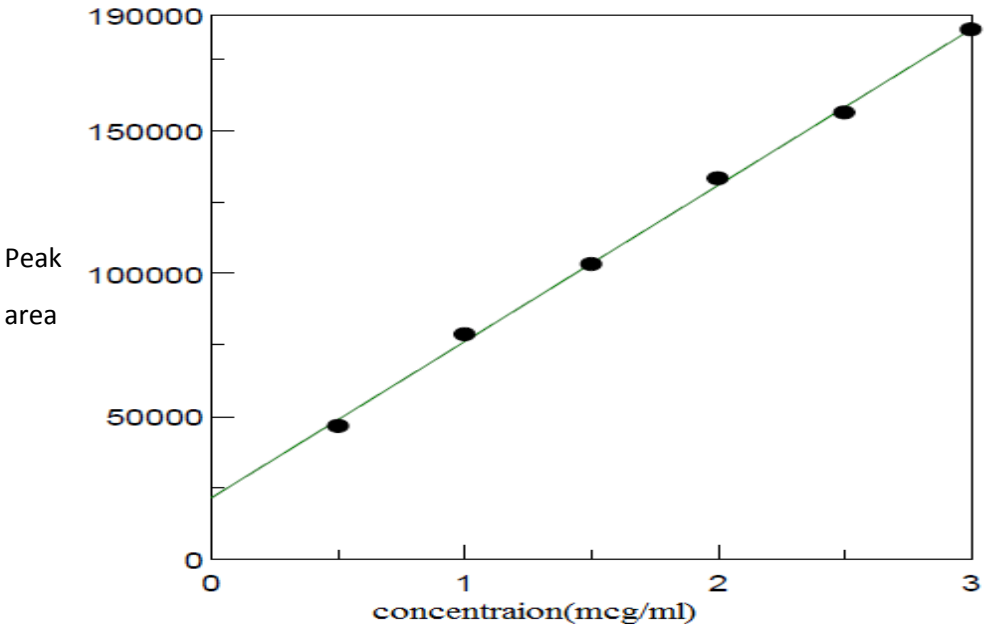


Fig. 44 chromatogram of brivudine (0.5µg/ml)

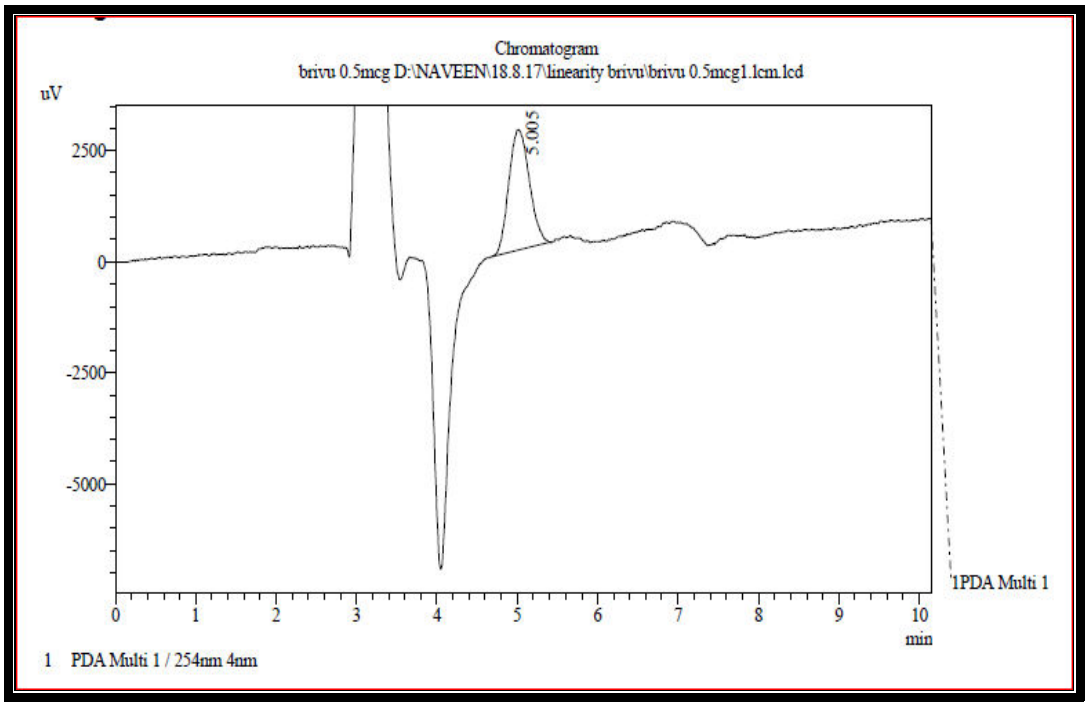


Fig. 45 chromatogram of brivudine (1 µg/ml)

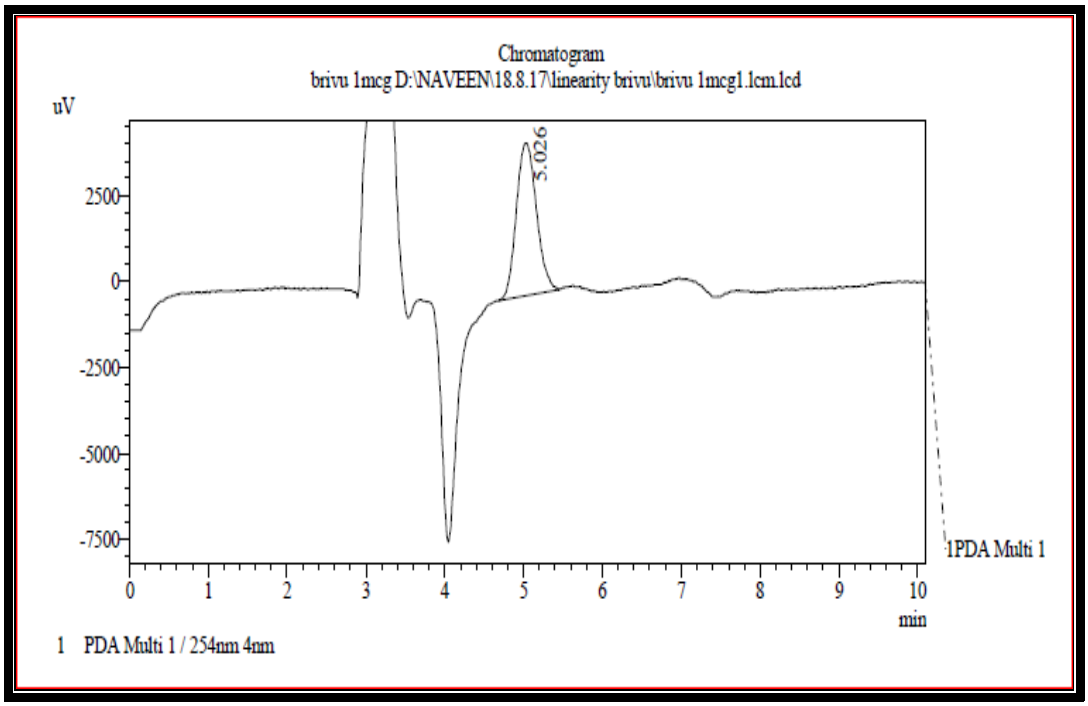


Fig. 46 chromatogram of brivudine (1.5µg/ml)

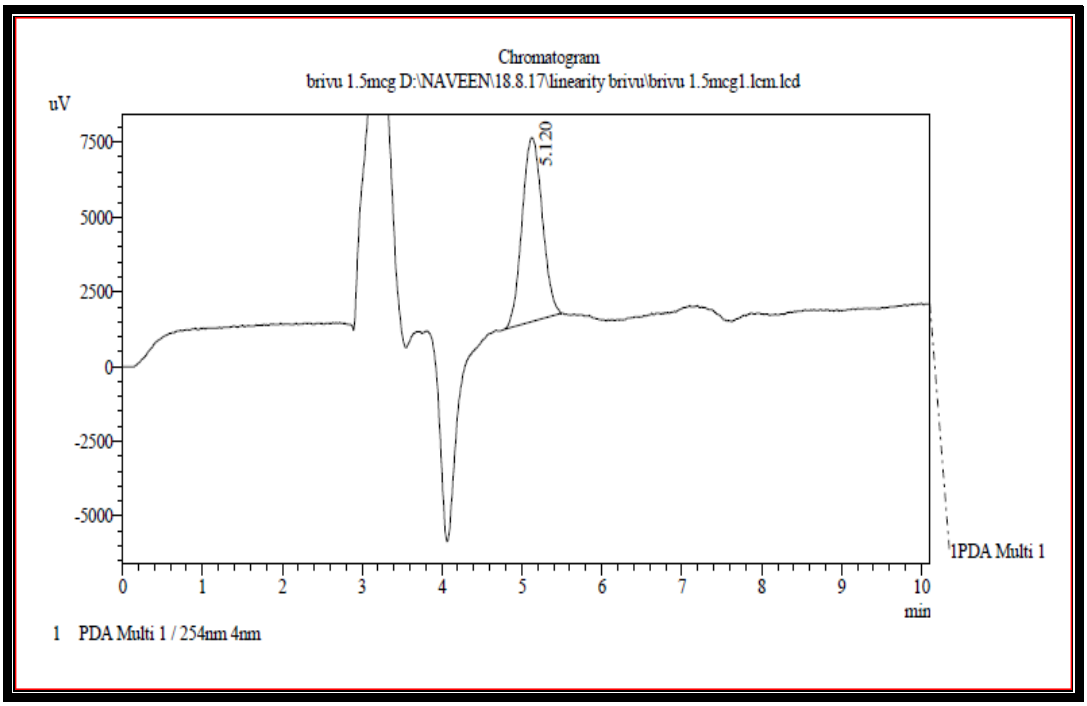


Fig. 47 chromatogram of brivudine (2µg/ml)

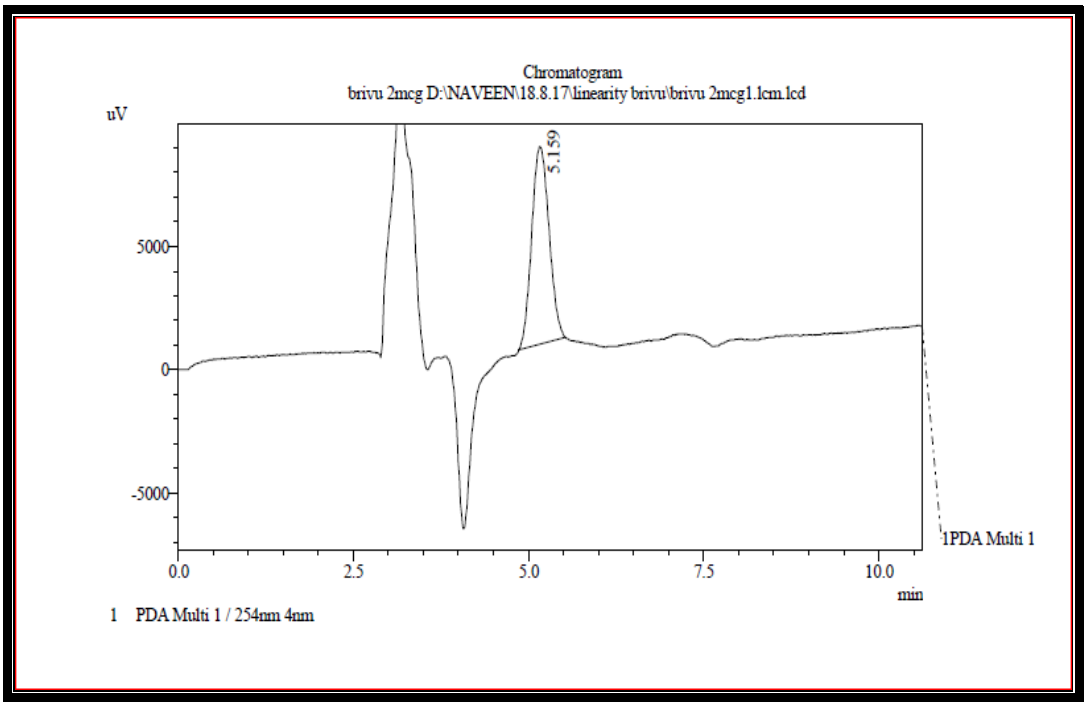


Fig. 48 chromatogram of brivudine (2.5µg/ml)

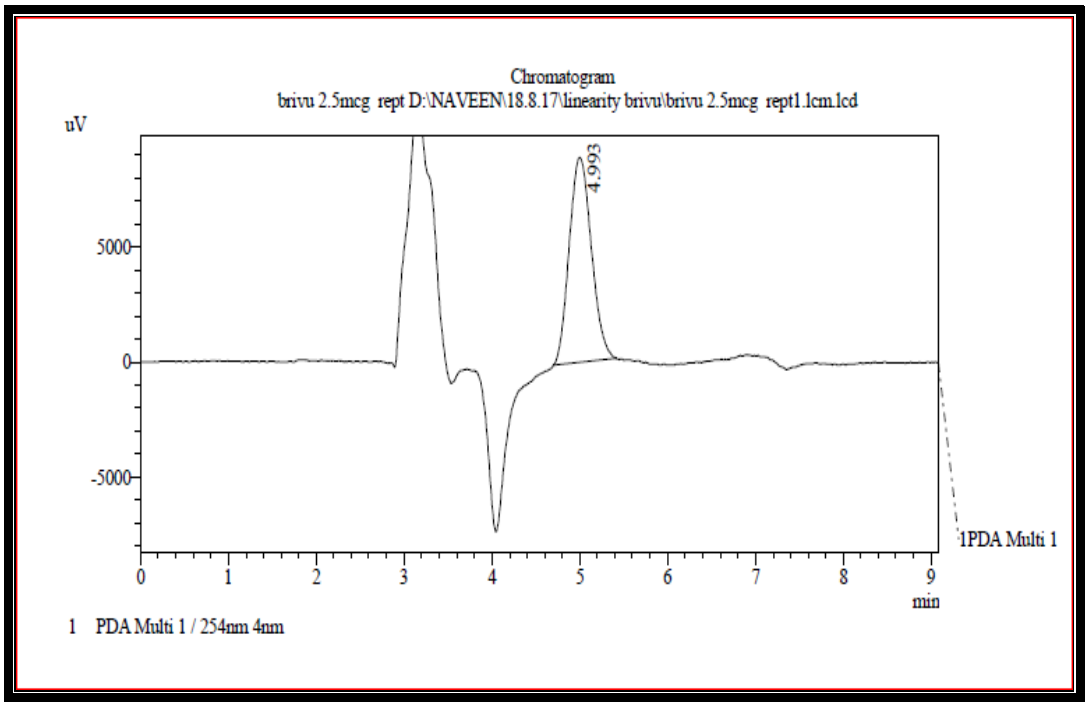
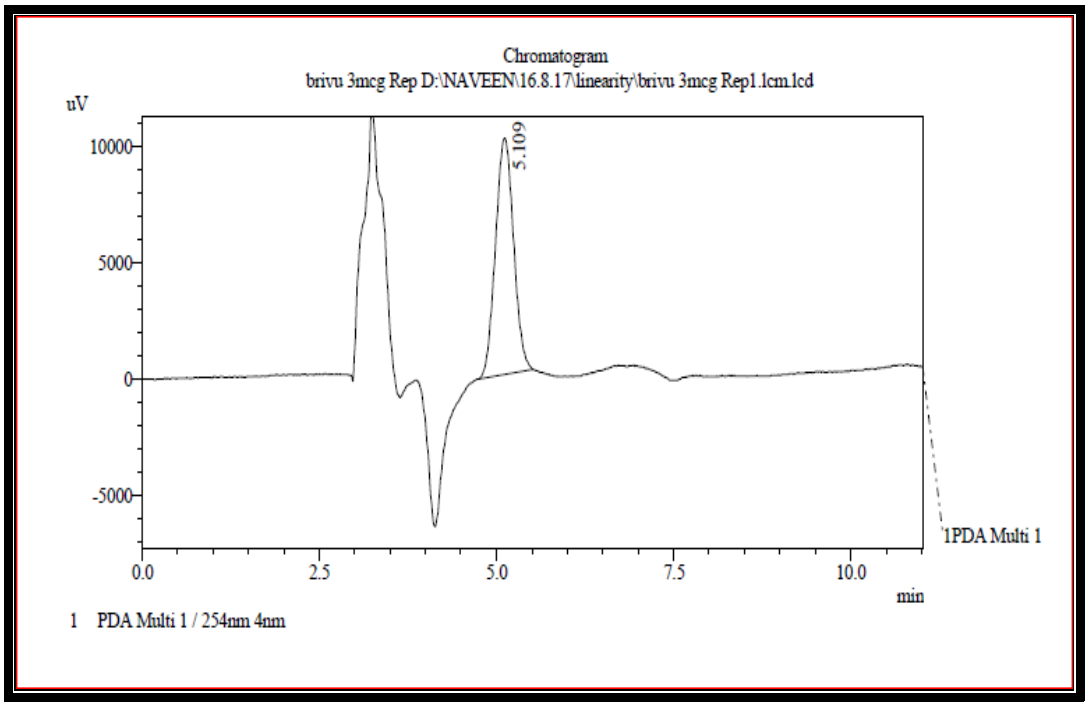


Fig. 49 chromatogram of brivudine(3 µg/ml)



Precision:

Intraday precision was determined by injecting standard solutions in between linearity range (1.5 & 2mcg/ml) were injected three times on the same day and % RSD was calculated (table 31).

Interday precision:

Interday precision was determined by injecting standard solution in between linearity range (1.5&2mcg/ml) were injected for three days and % RSD was calculated table 31.

Repeatability:

Repeatability of injections was determined by injecting standard solutions 1mcg/ml for six times, noted peak area and % RSD was calculated table 32.

Table 31: Intraday and interday precision

Concentration(mcg/ml)	Peak area		%RSD	
	Intraday	Interday	Intraday	Interday
1.5	107980	109350	0.7	0.9
	106476	107314		
	107553	108978		
2	130824	130824	0.9	1.0
	132389	128418		
	129636	128567		

Table 32: Repeatability sample measurement and sample application

Concentration (mcg/ml)	Peak area		%RSD	
	Sample measurement	Sample application	Sample measurement	Sample application
1	78568	78565	0.7	0.8
	77792	77982		
	77129	77129		
	78234	78234		
	78564	79001		
	78444	77895		

Limit of detection and limit of quantitation:

The LOD and LOQ were found to be 0.3mcg/ml and 0.5mcg/ml respectively.

Specificity:

There were no additional peaks observed while injecting solvents or mobile phase alone. The peak purity index of standard brivudine was 0.9997. This proves specificity of the method.

Robustness:

In order to demonstrate the robustness of the method, the following optimized conditions were slightly changed.

- ±0.2ml flow rate
- ±0.5% organic solvent
- ±0.1pH

Stability of solution:

The solution under room temperature was stable for 24 hour and up to 72hr stable under refrigeration.

System suitability studies:

The system suitability parameters like peak area, tailing factor, theoretical plate count, resolution, and retention time were calculated from the standard chromatograms table 33.

Table 33: system suitability parameters

Drug	Theoretical plate (N)	Retention time (min)	Tailing factor
Brivudine	1963.8	5.1	0.9

Analysis of formulation

Twenty tablets each containing 125mg of brivudine was taken for the study and average weight was determined. Quantity to 10mg brivudine was weight and transferred to 100ml standard flask and it was dissolved and made up to volume with methanol. Further dilutions were made and injected. The chromatogram is shown in fig 49. The result of analysis of formulation is shown in table 34. The peak purity index was found to be 0.9991.

Fig. 50 chromatogram of brivudine (1 µg/ml)

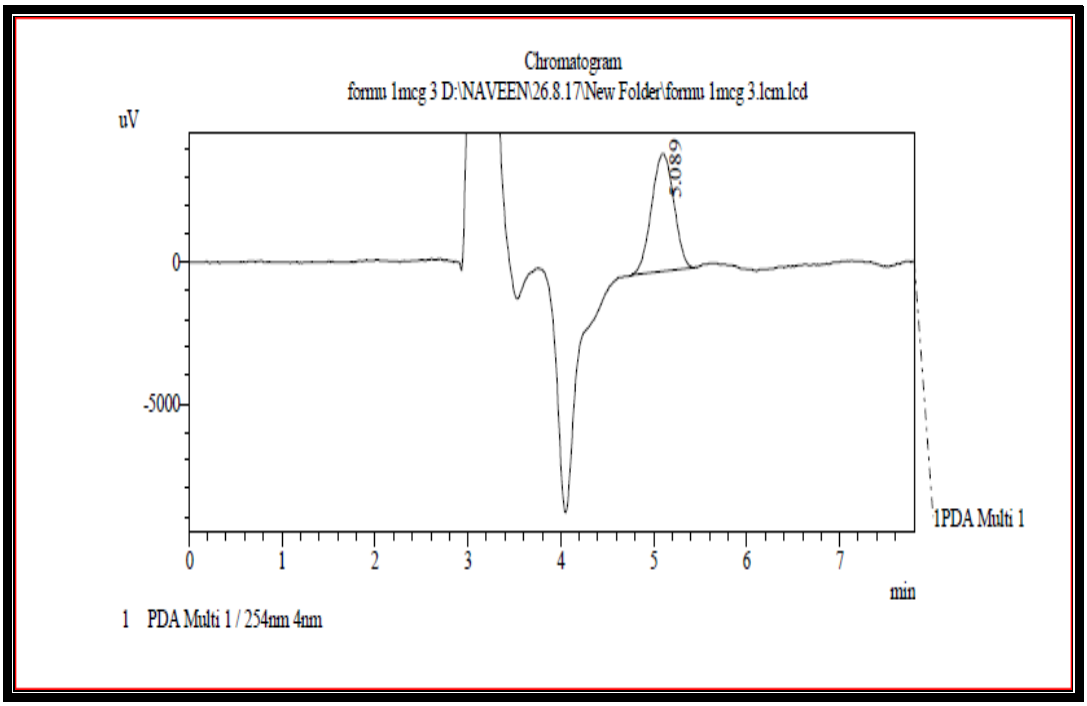


Table 34: analysis of formulation:

Brand	Amount of drug/tablet		% label claim	% RSD*
	Label(mg)	**found(mg)**		
Zostex	125	119	95.2%	1.2

*Average of six observation

Recovery study:

Recovery studies were carried out at 80% and 120% levels. The percentage recovery and % RSD of the results were calculated table 35. The high % recovery values shows accuracy of the method.

Table 35: Recovery studies

Level	**% Recovery**	**% RSD***
80	98.9%	0.82
120	99.12%	0.68

*Average of six observation

DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD FOR THE ESTIMATION OF BRIVUDINE IN HUMAN PLASMA

A HPLC method was developed and employed for employed for bioanalytical estimation of brivudine from human plasma.

The chromatographic conditions are the mobile phase consist of 1% O-phosphoric acid : methanol(40:60 % v/v), stationary phase is C_{18} and detection wavelength at 254nm. The RT of brivudine was observed at 5.1min.

Selection of internal standard:

In the fixed chromatographic conditions acyclovir and sofosbuvir were injected for the selection of internal standard. Sofosbuvir was selected as internal standard, it was well resolved from drug peak with good peak characters. The chromatograms are shown in fig 51 & 52.

Fig.51 chromatogram of acyclovir

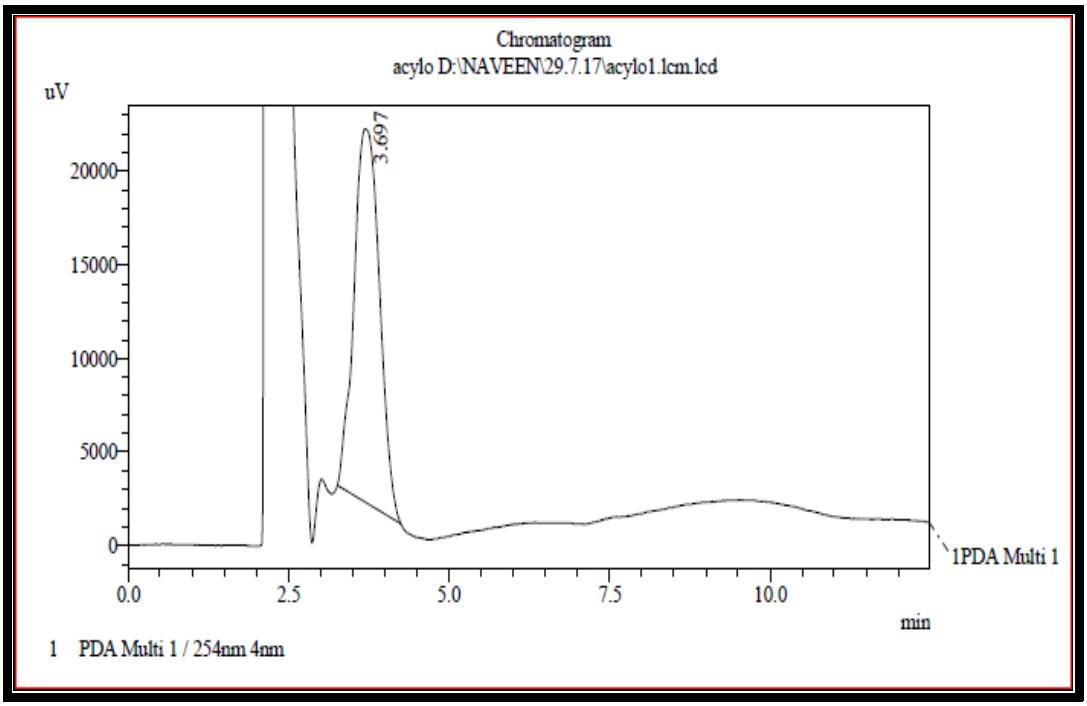
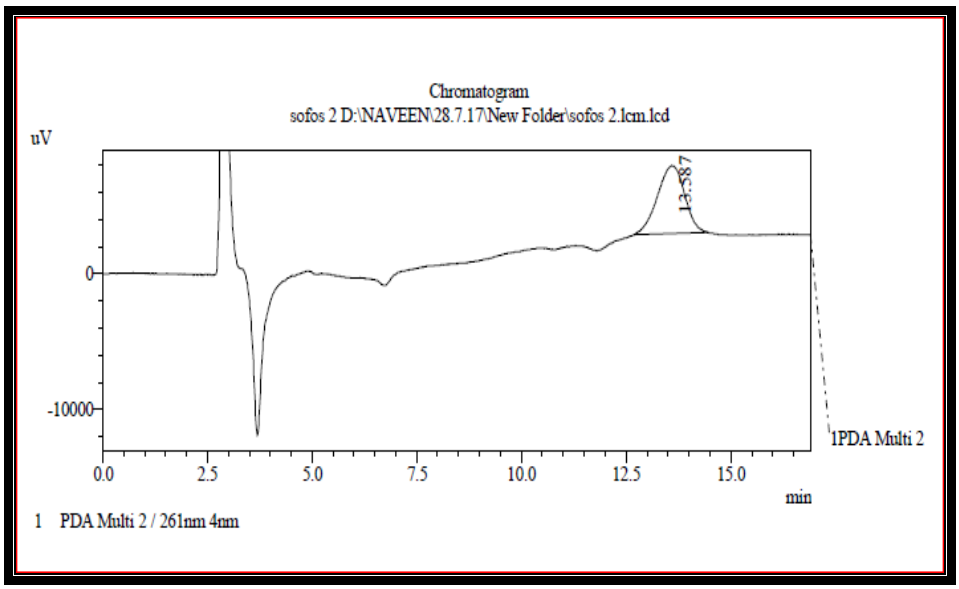


Fig.52 chromatogram of sofosbuvir



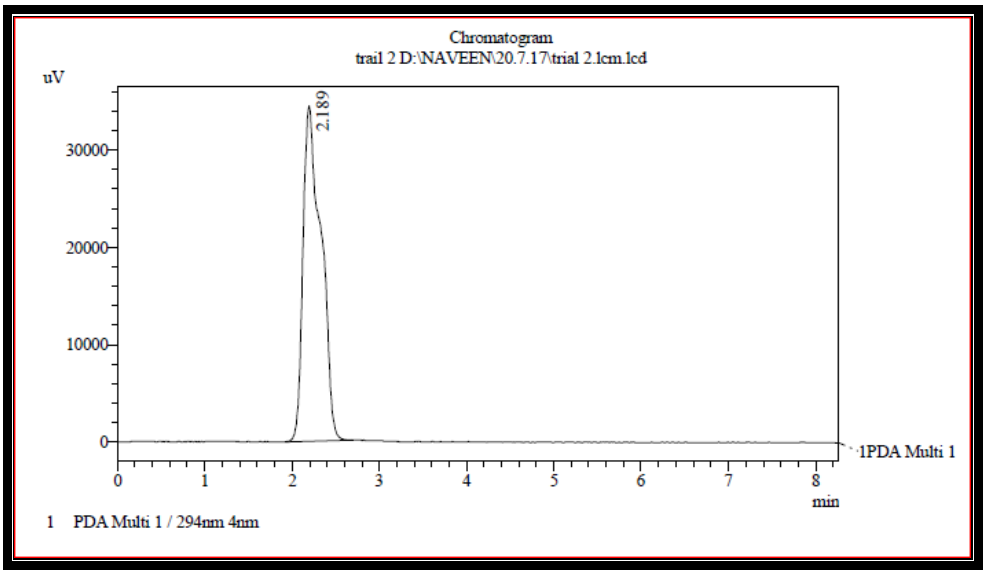
Optimization of extraction procedure:

To extract brivudine from plasma, protein precipitation method was tried using methanol and DMSO. Different volume of each organic solvent like 0.5ml, 1.0ml was tried for better recovery. The extraction efficiency was good with methanol when compared to all trials. The high recovery was obtained using methanol as an extraction solvent and no interfering peaks were observed at the retention time of brivudine and internal standard.

Specificity:

Specificity was demonstrated by blank plasma samples and plasma spiked with internal standard. They were extracted using the above procedure and chromatogram recorded. It was found that there was no interference from blank plasma at the retention time of brivudine (5.1min) and internal standard (11.2) were shown in Fig.53.

Fig.53 Chromatogram of Blank plasma



Linearity and range:

Linear regression data revealed an excellent linear relationship between concentration and peak area ratio. The retention time of brivudine was 5.1 min and internal standard 11.2min. The quantity of internal standard spiked was 100µl. A calibration curve has shown the linearity in the concentrations range of 0.5mcg/ml-3mcg/ml with correlation coefficient of 0.9245. The slope, and intercept, values -0.00639913 and 0.271063 respectively. The calibration graph is shown in fig 54 and calibration data in table 36. The standard chromatogram of plasma spiked with brivudine is shown in fig 55-60.

Fig. 54 Calibration graph

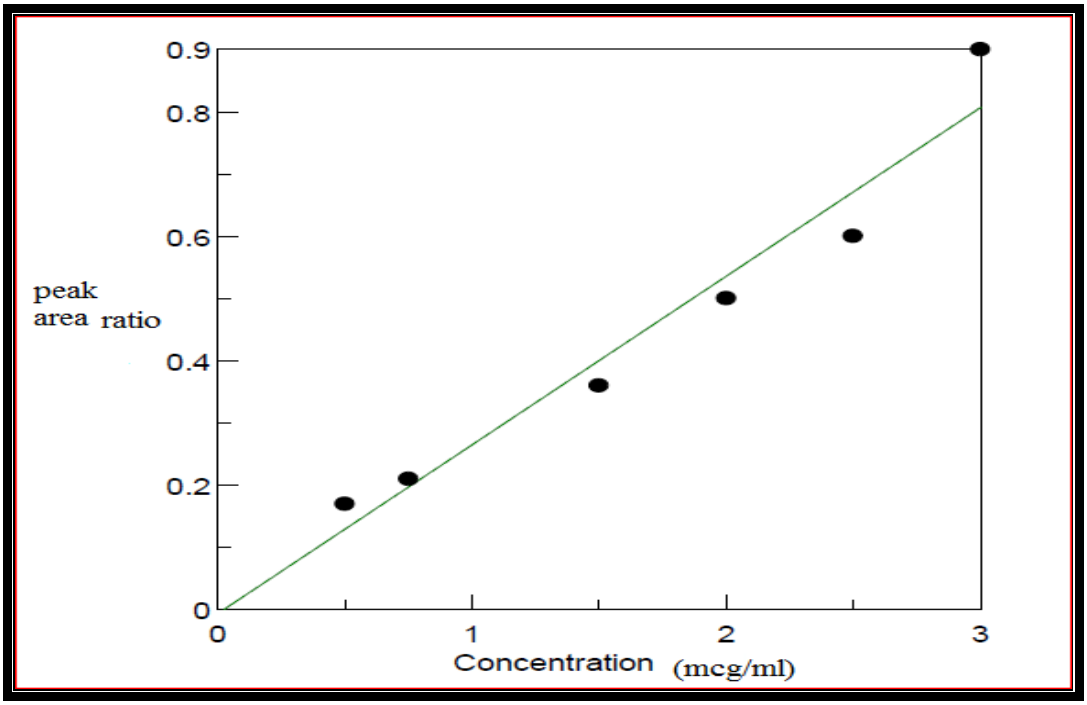


Fig.55 chromatogram for brivudine 0.5mcg/ml

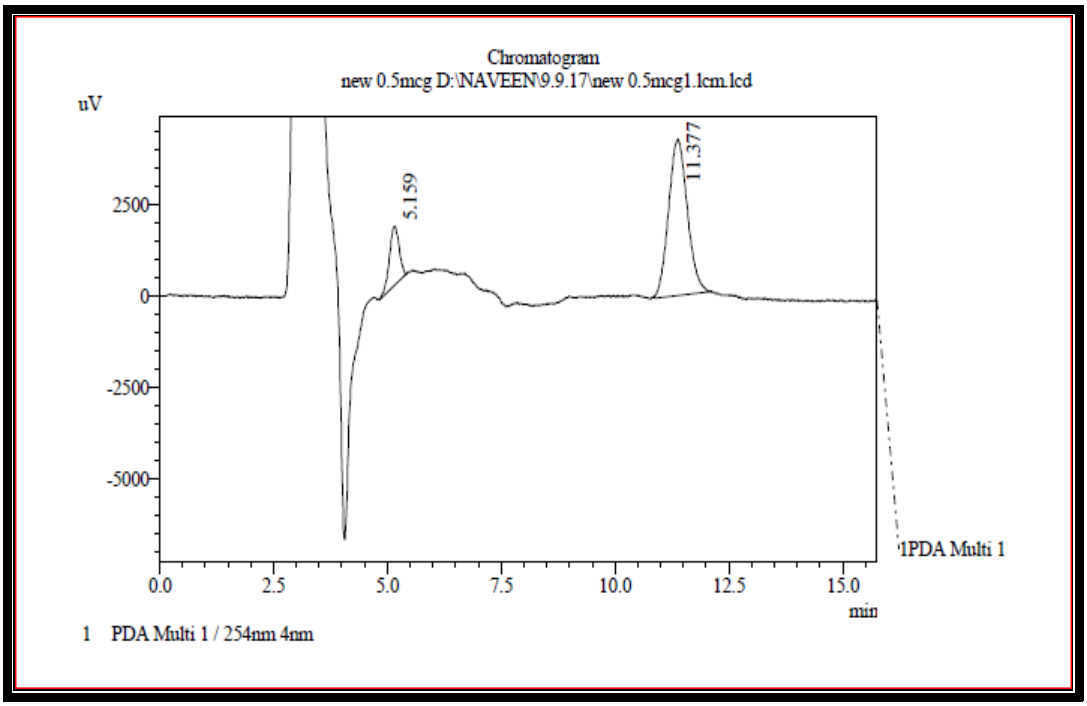


Fig. 56 Chromatogram for brivudine 0.75mcg/ml

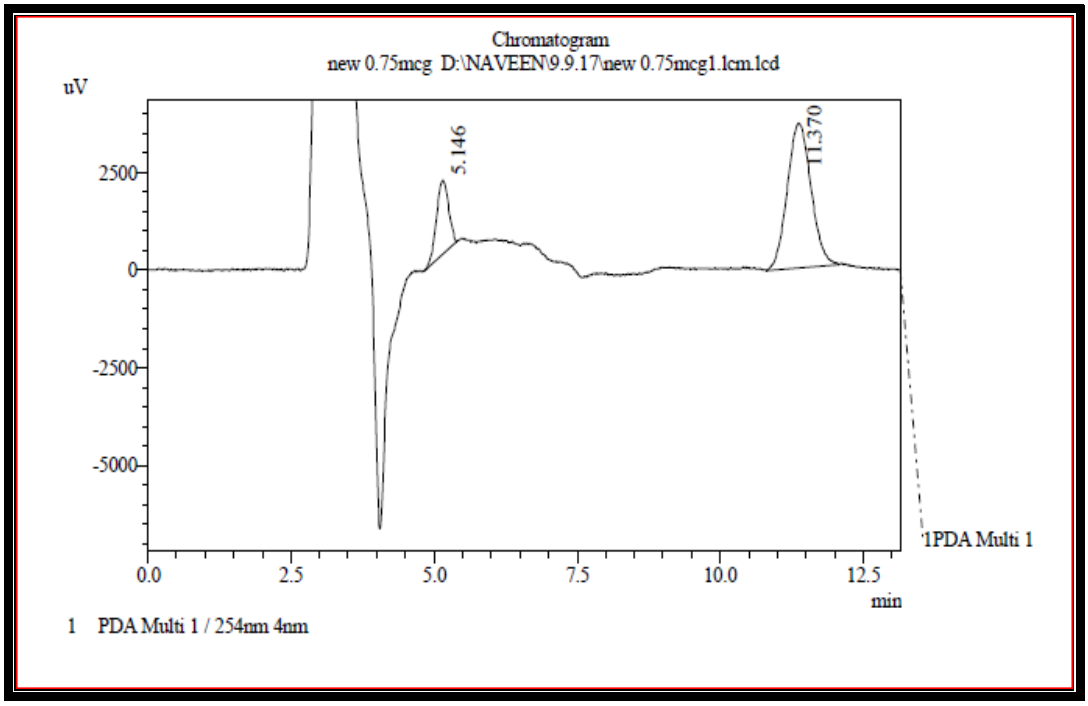


Fig.57 chromatogram for brivudine 1.5mcg/ml

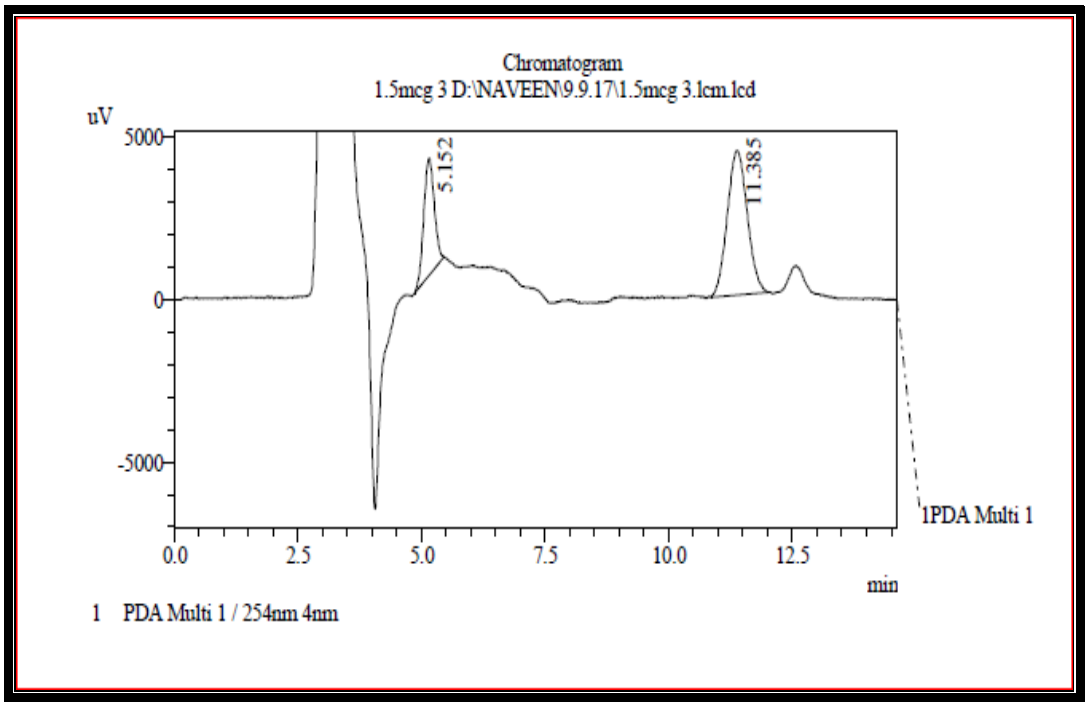


Fig.58 chromatogram for brivudine 2mcg/ml

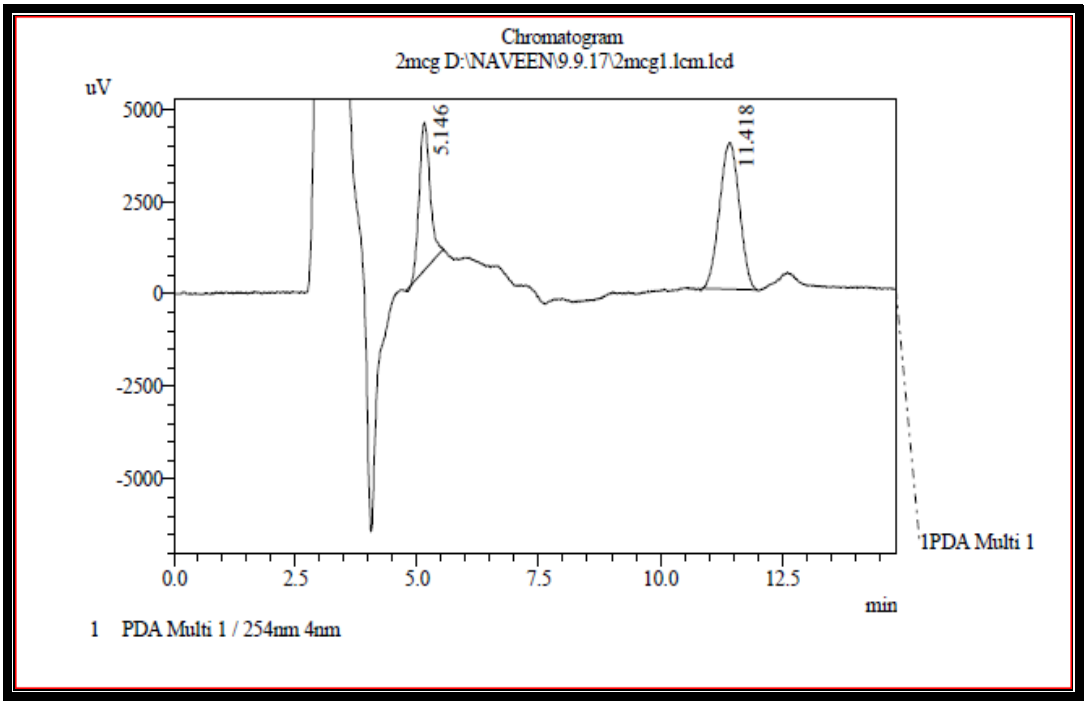


Fig.59 chromatogram for brivudine 2.5mcg/ml

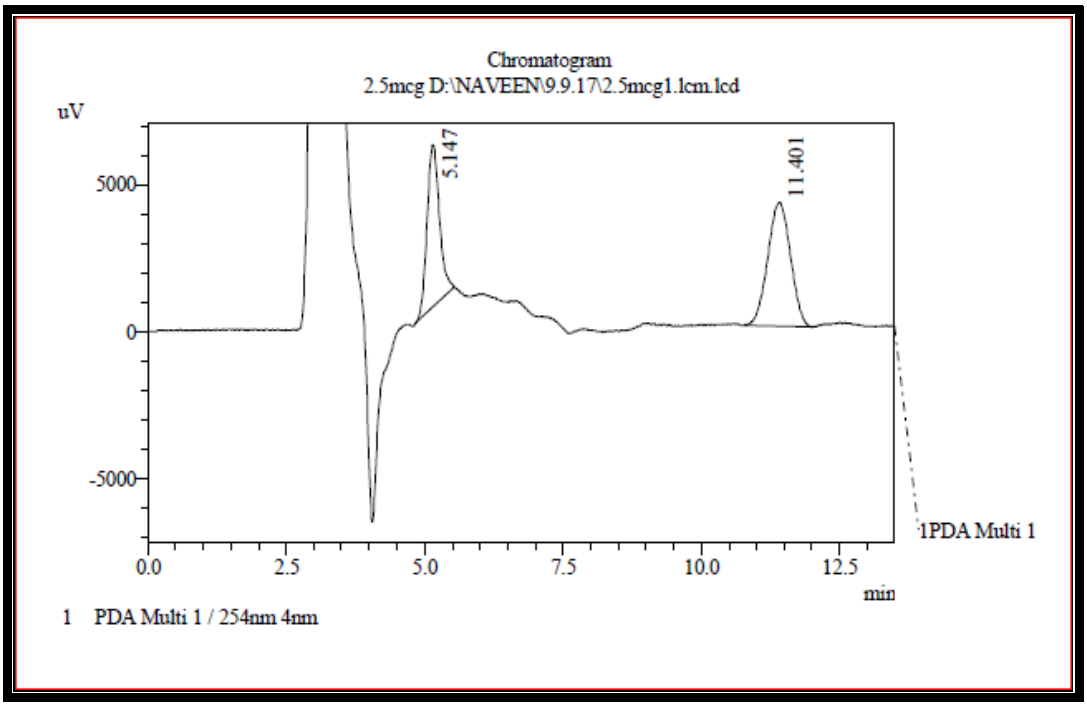


Fig.60 chromatogram for brivudine 3mcg/ml

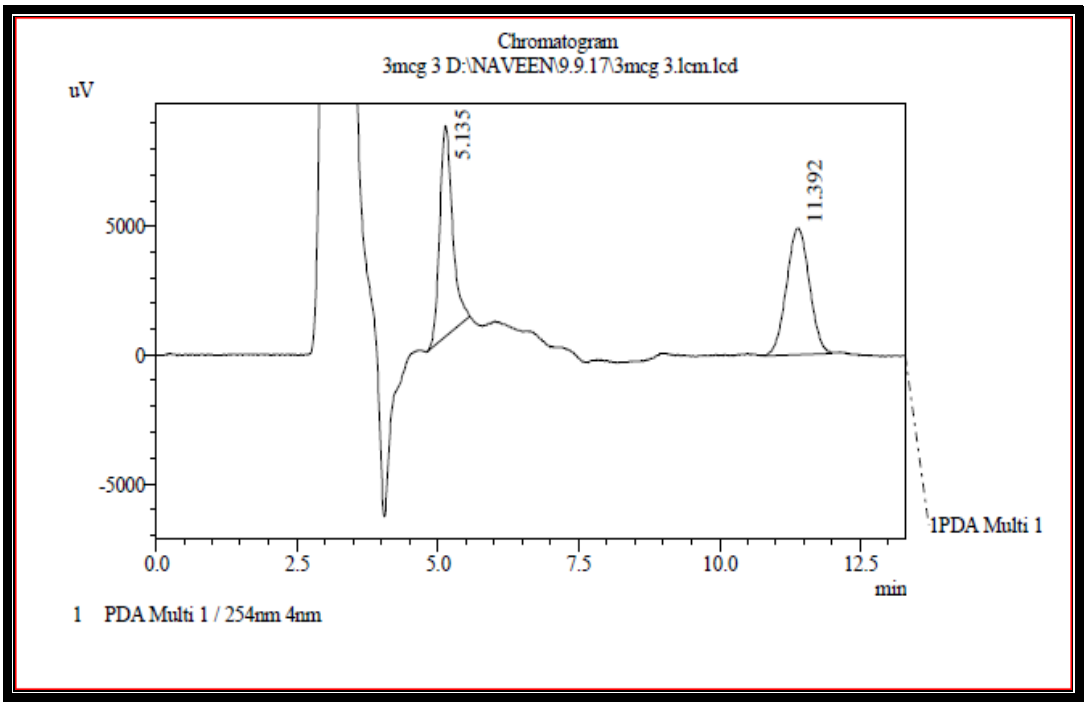


Table.36 Calibration table of brivudine (Bioanalytical method)

Concentration (mcg/ml)	Concentration of internal standard	Peak area		Peak area ratio (brivudine/ Sofosbuvir(IS))
		brivudine	sofosbuvir	
0.5	**10mcg/ml**	22427	129038	0.17
0.75		28034	106577	0.26
1.5		44290	131524	0.36
2		61794	122505	0.50
2.5		85934	124255	0.69
3		126013	132325	0.95

Accuracy:

Accuracy was determined by calculating the ratios of the predicted concentration to spiked values and with the precision express as %RSD. The mean value should be within 15% of the nominal value except at LLOQ, MLOQ, HLOQ where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy (table 37). The values are obtained with in the deviation limit which proves the accuracy of the method.

Table 37 Accuracy study

True concentration (mcg/ml)			Peak area*			% deviation from true value			Limit of % deviation
LLOQ	MLOQ	HLOQ	LLOQ	MLOQ	HLOQ	LLOQ	MLOQ	HLOQ	
0.5	1.5	3	22427	44290	126013	4.2	2.3	1.2	15
			24589	53432	128511				
			26598	41458	128937				

***Average of three observations**

Recovery:

Recovery reflects the degree of extraction. In general recovery is impacted by the interaction of the analyte with endogenous and/or exogenous components of matrix. The peak was compared with that of unextracted standard peak areas. The peak area response obtained was used for determining extraction efficiency (table 38). The % recovery was more than 50% on every occasion at three different levels.

Table 38 Recovery study

Concentration (ng/ml)	Mean % recovery*
500 (LLOQ)	51%
750 (MLOQ)	69%
3000 (HLOQ)	66%

***Mean of six observations**

Precision:

Intraday precision and inter-day precision:

Intraday and inter-day precision were estimated by analyzing five replicate samples at each concentration on the same day and on five consecutive days. Analysis of standard drug at three different concentrations in the linearity range (low, middle, high) and coefficient of variation was calculated (table 39 & 40).

Table. 39 Intraday precision

Concentration (mcg/ml)	Coefficient of variation (CV)*	Limit
0.5	9.4	Within 15%
1.5	5.2	
3.0	4.9	

***Mean of three concentrations**

Table. 40 Inter-day precision

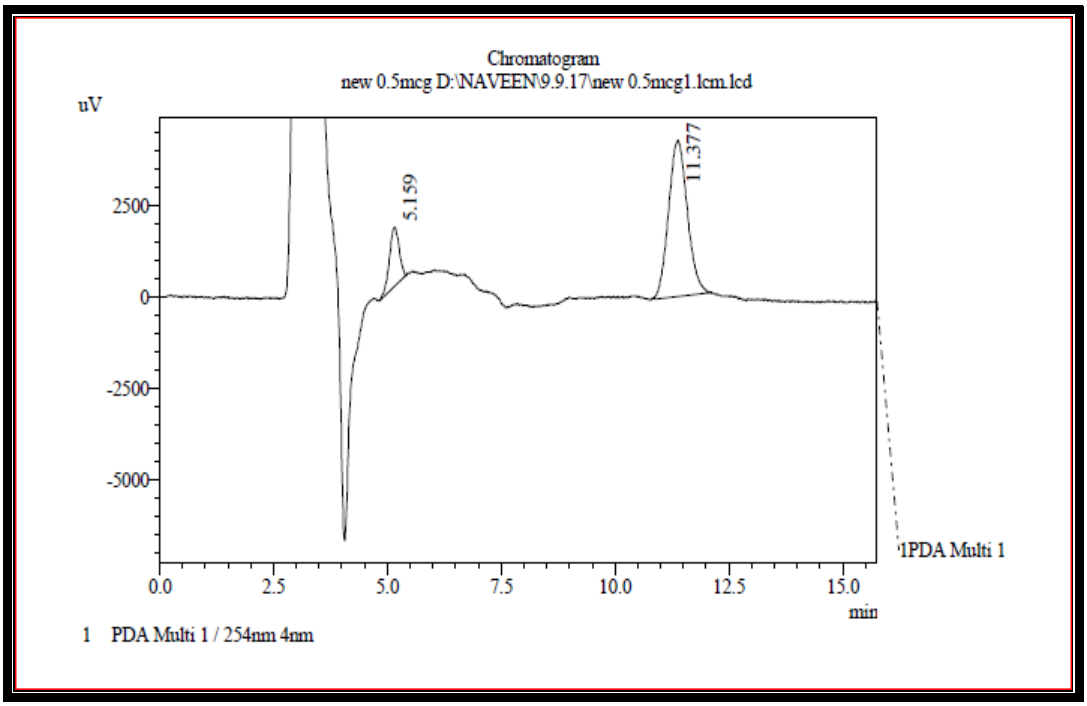
Concentration (mcg/ml)	Coefficient of variation (CV)*	Limit
0.5	8.5	Within 15%
1.5	6.9	
3.0	11.6	

***Mean of three concentrations**

Lower limit of quantitation:

The lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy fig 61.

Fig.60 Lower limit of concentration 0.5mcg/ml



Stability:

The stability of the drug was studied under the room temperature. The stability of brivudine is 6hrs in room temperature (after extraction).

SUMMARY AND CONCLUSION

In the present study 3 cephalosporin class of drugs (cefixime, cefpodoxime, cefepime) and an antiviral drug brivudine were selected and HPTLC methods were developed and validated.

One RP-HPLC method was also developed and validated for brivudine, and the developed HPLC method was applied to estimate the drug in plasma.

ANALYTICAL METHOD DEVELOPMENT:

HPTLC METHOD:

For the determination of cefixime by HPTLC method, a system comprising of ethyl acetate: methanol: water (4.5: 5: 0.5 % v/v/v) was selected because this system gave good separation with symmetric peak (Rf = 0.58) at the selected wavelength of 292nm. Calibration graph was plotted in the concentration range of 200-450ng/band(r = 0.998). The developed plate was stable upto 8 hours. Limit of detection was found to be 40ng/band and limit of quantification was found to be 100ng/spot. This method was successfully applied for the determination of cefixime from tablet dosage form.

For the estimation of cefpodoxime by HPTLC, different mobile phase systems were tried with different ratios. It was found that a system comprising of a mobile phase system containing Methanol: ethyl acetate: toluene (1.5: 3: 3.5 v/v/v) was selected because in this system the drug solutions gave better peak with good resolution and also offered an optimum migration (R_f = 0.53) at the selected wavelength of 280nm. Linearity was found over the concentration range of 100-300ng/band (r = 0.999). The slope, intercept and correlation coefficient values were found to be 8.1124, 293.581 and 0.999 respectively.

For the determination of cefepime by HPTLC method, a system

comprising of chloroform: methanol: water (1: 6: 3 % v/v/v) was selected because this system gave good separation with symmetric peak (Rf = 0.44) at the selected wavelength of 285nm. Calibration graph was plotted in the concentration range of 500-2500ng/spot(r = 0.998). The developed plate was stable up to 8 hours. Limit of detection was found to be 120ng/spot and limit of quantification was found to be 500ng/spot. This method was successfully applied for the determination of cefepime from tablet dosage form.

In the developed HPTLC method brivudine stock solution of 100mcg/ml was prepared in methanol. The standard solution and formulation were spotted on pre coated silica gel G $60F_{254}$ on aluminum sheets which was followed by development using methanol: chloroform: toluene (2:5:3 v/v/v) and scanning at 301nm. The drug gave good symmetrical peak at R_f value of 0.50. It showed a linearity range in concentration 50-300ng/band. The slope, intercept and correlation coefficient values were found to be 19.87, 623.025 and 0.997 respectively.

Parameters	HPTLC		
	cefixime	cefpodoxime	cefepime
Linearity	200-450ng/spot	100-300ng/spot	500-2500ng/spot
LOD	40ng/spot	60ng/spot	120 ng/spot
LOQ	100ng/spot	100ng/spot	250ng/spot
Detection wavelength	292nm	280nm	285nm
% Label claim	97.6	97.5	99.7
Theotrical plate	38mm	42mm	39mm
Asymmetric factor	1	1.1	1

RP-HPLC METHOD FOR BRIVUDINE:

In the developed RP-HPLC method, optimization of the several chromatographic parameters like selection of mobile phase, detection wavelength, ratio of mobile phase and flow rate etc were done. A mobile phase system consisting of 1% ortho phosphoric acid (pH 6.5): Methanol (40:60 v/v) was selected for the separation of brivudine. With this system a peak with good resolution and less peak tailing was obtained at a flow rate of 0.8ml/min. The method was validated as per ICH guidelines.

Calibration curve was plotted using concentration (x) versus peak area(y). Linearity of brivudine was found over the range of 0.5 – 3mcg/ml. The correlation coefficient value was found to be 0.9995.

Bioanalytical method development:

A RP-HPLC method was developed for bioanalytical estimation of brivudine from human plasma. A mobile phase system consisting of 1% ortho phosphoric acid (pH 6.5): Methanol (40:60 v/v) was selected for the separation of brivudine & sofosbuvir (RT – 5.1 & 11.2) and detection was done at 254nm. The method was validated over the range of 0.5-3mcg/ml. The LLOQ is found to be 0.5mcg/ml. Intraday and interday precision studies were also carried out and variation was less than 15% & within the prescribed limit.

CONCLUSION

Cephalosporin class of drugs were selected and analytical methods

(HPTLC) were developed for its estimation. The HPTLC methods developed were validated as per ICH guidelines and found to be specific, accurate, and precise.

The method developed for the cephalosporin (cefixime, cefpodoxime, cefepime) can be used in industry for routine analysis to check the amount of the drug present in pharmaceutical dosage form.

Brivudine is an antiviral drug which was selected and analytical methods (HPTLC & HPLC) were developed for the determination of brivudine in bulk, pharmaceutical dosage form and in plasma.

The bioanalytical method developed is simple and showed good accuracy, specificity and reproducible. It can be used for the estimation of Brivudine in biofluids. The chromatogram developed was well resolved peak of Brivudine without any interference. The developed method could be applied in bioequivalence and pharmacokinetic studies.

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